

**THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS IS A CENTRAL
MEDIATOR OF ASTHMA PATHOGENESIS**

by

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The receptor for advanced glycation end products (RAGE) is a multiligand, cell surface receptor. Isolated from lung, the organ in which it is most abundant, RAGE has since been shown to be a pro-inflammatory mediator in the pathogenesis of diabetes, atherosclerosis, rheumatic and neurodegenerative syndromes, and cancer. However, despite its localization, the role of RAGE in asthma and allergic airway disease is largely unknown.

The studies described herein explore RAGE's effect on disease phenotype using several different models of allergic airway disease/asthma in mice, with house dust mite extract or ovalbumin as the provoking allergen. Respiratory mechanics were assessed using mechanical ventilation and the forced oscillation technique; bronchovascular architecture and airway remodeling were evaluated using general histochemical stains. Expression of RAGE, immunoglobulin, and relevant cytokines was characterized by standard protein detection methods and/or real-time PCR, while immunohistofluorescence microscopy was used to determine the cellular localization of proteins of interest.

In both house dust mite and ovalbumin models of chronic allergic airway disease/asthma, the absence of RAGE abolishes most assessed measures of pathology, including airway hypersensitivity (resistance, tissue damping, and elastance), eosinophilic inflammation, and mucus hypersecretion. IL-17 demonstrates complex regulation, with elevated baseline expression in RAGE knockouts, but no induction in response to allergen challenge. IL-4 and IL-25

secretion, immunoglobulin isotype class switching and antigen recognition are intact in the absence of RAGE. In contrast, allergen-induced up-regulation of eotaxin, eotaxin-2, IL-5, IL-13, and IL-33 is abrogated in RAGE's absence.

RAGE's soluble isoform is a decoy receptor that impedes ligand binding to the membrane isoform and thus downstream signaling. Soluble RAGE (sRAGE) has been efficacious in ameliorating disease in a broad array of models in which the membrane RAGE isoform is thought to be involved. Because few studies have explored the use of this agent in the lung, clearance and biodistribution studies were performed, exploring three common routes of administration; intratracheal delivery was found to be substantially superior to other modes. sRAGE was used as a therapeutic in an animal model of asthma and was shown to markedly reduce inflammation, suggesting that inhibition of RAGE may serve as a promising therapeutic strategy.

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PREFACE

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ABBREVIATIONS

AGE	advanced glycation end product	KO	(e.g. interleukin-4: IL-4) knockout
AHR	airway hyperreactivity	LPS	lipopolysaccharide
alum	aluminum hydroxide	MAPK	mitogen-activated protein kinase
ANOVA	analysis of variance	MBP	major basic protein
AQP5	aquaporin 5	MHC	major histocompatibility complex
α -SMA	α -smooth muscle actin	MSA	mouse serum albumin
BALF	bronchoalveolar lavage fluid	NBF	neutral buffered formalin
BM	bone marrow	NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
BSA	bovine serum albumin	NFAT	nuclear factor of activated T-cells
CCSP	Clara cell secretory protein	OVA	ovalbumin
COPD	chronic obstructive pulmonary disease	PAS	periodic acid-Schiff
DAMP/		PBS	phosphate buffered saline
PAMP	damage/pathogen associated molecular pattern	PECAM-1	platelet endothelial cell adhesion molecule 1, CD31
DTT	dithiothreitol	proSP-C	pros surfactant protein C
ELISA	enzyme-linked immunosorbent assay	PRR	pattern recognition receptor
emPAI	exponentially-modified protein abundance index	PSGL-1	P-selectin glycoprotein ligand
ERK	extracellular-signal-regulated kinase	PVDF	polyvinylidene fluoride
esRAGE	endogenous secretory RAGE	qRT-PCR	quantitative reverse transcription polymerase chain reaction
FEV(1)	forced expiratory volume in 1 s	(m)RAGE	(membrane) receptor for advanced glycation end products
FVC	forced vital capacity	Rn	Newtonian resistance
G	tissue damping	S.E.M.	standard error of the mean
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
GWAS	genome-wide association study	SNP	single-nucleotide polymorphism
H	elastance	sRAGE	soluble RAGE
H&E	hematoxylin and eosin	TSLP	thymic stromal lymphopoietin
HDM	house dust mite	VCAM-1	vascular cell adhesion molecule 1
HMGB1	high mobility group protein B1	WT	wild type
i.n.	intranasal		
i.p.	intraperitoneal		
i.t.	intratracheal		
i.v.	intravenous		
IgE	Immunoglobulin E		
IgG ₍₁₎	Immunoglobulin G (1)		
IL- <i>n</i>	Interleukin- <i>n</i>		

1.0 INTRODUCTION

What's in a name? When it dictates the first two decades of one's life, much apparently. Such is the story of the protein RAGE, whose name and circumstances of discovery have had a prevailing influence on the subsequent course of research. More than just a commentary on the extraordinary power of language, the history of RAGE research illustrates the triumph of biochemistry as a discipline and biochemical mechanism in particular over more archaic teleological thought patterns, within the modern biological research enterprise. Thus, it is RAGE's putative molecular interactions that have captivated the scientific discourse since its discovery in 1992; the organ in which it is predominantly expressed – the lung – and the obvious implications with regard to pulmonary homeostasis and pathophysiology, have been neglected as tangential details. The studies set forth herein seek to redress this imbalance, as it were, by shedding light on the role of RAGE in one of the most important diseases of the lung: asthma.

1.1 RAGE BIOLOGY

1.1.1 RAGE structure and function

The receptor for advanced glycation end products (RAGE) is a multiligand receptor of the immunoglobulin superfamily of surface-expressed receptors.¹ The discoverers of RAGE

identified it as a 35 kDa advanced glycation end product-binding protein isolated from bovine lung, but noted that expression of RAGE from cDNA transfected into cell lines demonstrated a 50 kDa protein. They attributed the discrepancy to multiple isoforms and variable post-translation glycosylation. The extracellular domain is composed of three immunoglobulin-like domains: V, C1, and C2, named for their resemblance to the corresponding variable and constant domains of immunoglobulin.¹ The extracellular domain, and specifically the V-C₁ functional domain (which acts as single unit²), is responsible for ligand binding.³ A single hydrophobic transmembrane domain links the ligand-binding extracellular portion to a cytoplasmic domain, which is essential for downstream signal transduction.⁴⁻⁶ RAGE is glycosylated at two asparagine residues,⁷ a property known to be critical in RAGE ligand binding and function.^{3,8,9} In view of this, it is worth pointing out at this juncture that unfortunately many of the biochemical studies of RAGE have been performed on protein expressed in bacteria, yeast, or insect cells, in which glycosylation is either absent or at any rate markedly less complex than in higher eukaryotes.

RAGE comes in two flavors: a membrane isoform (mRAGE) and essentially two soluble isoforms. The soluble isoforms include the entire extracellular portion of the protein (thus inheriting the ligand binding capacity of the membrane isoform), while lacking the transmembrane and cytosolic domains. Soluble isoforms of RAGE may arise via alternative pre-mRNA splicing¹⁰ (there may be some variability in splicing, such that multiple products are possible) or proteolytic cleavage of the membrane isoform. The former is sometimes called endogenous secretory RAGE¹¹ (esRAGE) whereas the latter is named sRAGE, a term that sometimes subsumes all soluble RAGE isoforms. Matrix metalloproteinases have been found to mediate proteolytic cleavage of RAGE^{12,13}; the majority of soluble RAGE in the circulation is

sRAGE, the cleaved form. As alluded to previously, the cytoplasmic signaling domain is critical to RAGE's function as a pro-inflammatory receptor. Synthetic isoforms lacking this domain, termed DN-RAGE, act in a dominant negative fashion to inhibit the function of normal RAGE,⁴ as well as of any other receptors binding to RAGE's ligands. It is not surprising, therefore, that soluble RAGE isoforms (regardless of their origin as cleavage product or secreted protein), which of course do not possess a signal transduction apparatus, antagonize the function of membrane RAGE. sRAGE (in the sense of all soluble isoforms of RAGE) does this by sequestering pro-inflammatory ligands, thus acting as a decoy receptor. It is important to state here that, as many RAGE ligands are also ligands of other pro-inflammatory receptors, some effects of sRAGE may be attributable to antagonism of non-RAGE signaling axes.

Agonistic ligation of RAGE is believed to activate multiple signal transduction pathways, including, but not limited to, p38 mitogen activated protein kinase (MAPK),¹⁴ p42/p44 MAPK (extracellular-signal-regulated kinase 1/2 (ERK 1/2)),¹⁵ and p21 (Ras) MAPK.¹⁶ This eventuates in the release of NF- κ B from cytosolic sequestration and translocation into the nucleus to induce transcription of genes encoding pro-inflammatory mediators.¹⁷ RAGE activation by application of ligands frequently induces up-regulated RAGE expression, a positive regulatory feedback loop that may be rationalized by the existence of three putative NF- κ B-like binding sites in the promoter region of RAGE itself.¹⁸ Additionally, sustained RAGE activation induces increased transcription of the p65 subunit of NF- κ B, a phenomenon that amplifies the reserve of this pro-inflammatory transcription factor and thus the dynamic range of the downstream response.¹⁹

On the basis of history and overarching hypotheses, RAGE ligands may be partitioned into three categories. The first of these is comprised of the advanced glycation end products (AGEs), which continue to bear such a strong mark in the field of RAGE research by virtue of

historic primacy. To summarize briefly, AGEs are proteins or lipids with sugar moieties incorporated via non-enzymatic organic reactions, such as the Maillard reaction. While AGEs are normally generated endogenously at low levels, the hyperglycemic and oxidative state in diabetes promotes production of elevated levels of AGEs. Although receptors for the uptake of AGEs exist on phagocytic cells,^{20,21} it was only with the discovery of putatively endothelium-expressed RAGE that an elegant mechanism connecting elevated AGEs to vascular complications of diabetes was in place.¹ However, recognizing the implicit absurdity of a receptor whose apparent sole purpose is to lie in wait for diabetes to arise (with no obvious adaptive role in that disease), and perhaps because of considerations related to RAGE's molecular architecture, the authors suggested that AGEs may not be the natural ligands of RAGE. This set the stage for the ensuing years of research devoted to other RAGE ligands.

The main body of research on RAGE ligands explored RAGE's role as a pattern recognition receptor (PRR). PRRs were identified as the key sensors of the innate immune system, detecting microbial pathogens based on conserved molecular patterns, termed pathogen associated molecular patterns (PAMPs).²² PAMPs are general molecular motifs unique to pathogens (e.g. cell wall components in bacteria or viral-type nuclei acid), whose role is so fundamental to the pathogen's survival and propagation that mutation attributable to drift or selection pressure is practically impossible. In succeeding years, it was recognized that many sterile disease processes, such as tissue injury or infarction, involved a prominent inflammatory component. The postulated and later discovered endogenous mediators driving this response were termed damage associated molecular patterns (DAMPs).²³ These mediators may be released from necrotic cells by passive leakage (e.g. high mobility group protein B1 (HMGB1)) or secreted in a regulated fashion from inflammatory effector cells at sites of injury (e.g.

calgranulins released by neutrophils). It should be noted here that although amyloid β peptide (a known ligand and activator of RAGE²⁴) is not one of the classic DAMPs, it may be categorized with the latter in that it is frequently associated with a disease state.

HMGB1, also known as amphoterin, is constitutively expressed in the nucleus, where it facilitates nucleosome assembly. During necrosis (but not apoptosis) and in certain states of activation, HMGB1 is released into the extracellular space, where it binds and activates several pro-inflammatory PRRs, including Toll-like receptor 2 (TLR2), Toll-like receptor 4 (TLR4), and RAGE.^{25,26} The HMGB1-RAGE interaction has been implicated in a number of inflammatory disease processes, including neoplasm metastasis,²⁷ arthritis,²⁸ colitis,²⁹ and ischemia-reperfusion injury.³⁰ Even so, reflection on the fact that other HMGB1-binding receptors, such as TLR2 and TLR4, are bound and agonized or antagonized by a vast array of exogenous substances (from bacterial lipopolysaccharide (LPS) and fungal zymosan to measles hemagglutinin, opioid analgesics, and nickel ions), should lead one to accept the thesis that HMGB1 is the definitive ligand of RAGE with caution.

Members of the calgranulin family are also known to bind to RAGE and have been implicated in diseases and pathophysiologic phenomena such as cardiomyopathy,³¹ neurodegeneration,³² and tumor invasion.³³ Known also as S100 proteins, these small calcium-binding proteins are structurally similar to calmodulin, but in contrast to the latter they demonstrate a considerably narrower cell and tissue distribution. RAGE has been shown to bind and respond to S100A8/S100A9,^{9,34} S100B, S100A4, S100A6, S100A11, and S100A12.³⁵ As S100 proteins are named for their complete (100%) solubility in ammonium sulfate at neutral pH – which is a reflection of biochemistry rather than physiology – and as the distribution, expression pattern, and apparent function differs markedly for each member of the family, the

fact that so many bind to RAGE may be less indicative of true biological relevance of these interactions than of promiscuous binding owing to large, labile, or low-complexity interaction domains.

The last category of RAGE ligands is comprised of those largely ignored by the bulk of the research community. They have been investigated primarily by those who found RAGE's localization to the lung compelling, and wished to understand its homeostatic function better. Glycosaminoglycans such as heparan sulfate³⁶ and heparin³⁷ (whose affinity is exploited in RAGE purification) are among these, as are collagen I and IV and laminin.³⁸ RAGE has been observed to be important in mediating adherence and spreading of transfected cells to collagen-coated surfaces,³⁹ lending physiologic support to results obtained from *in vitro* binding studies, and suggesting that RAGE may be involved in adhering pulmonary epithelium to underlying basement membrane. Within this vein, RAGE has also been shown to act as a binding partner of the macrophage $\beta 2$ integrin Mac-1, which may indicate a role as a facilitator of leukocyte adhesion and trafficking.^{40,41}

Having briefly outlined some of the RAGE ligands, it difficult to discern how to go about determining the biological relevance of any particular interaction, as much of the work on RAGE ligands has been performed in rather artificial systems: mixtures of purified protein, cell lines over-expressing RAGE, administration of supraphysiologic quantities of exogenous protein, etc. Moreover, it may be a mistake in this case to give primacy to protein ahead of cell, tissue, and organ: it would be curious, indeed, to go about defining the primary role of collagen through enumeration of its many binding partners! To better understand RAGE function, its expression and localization must be considered.

1.1.2 RAGE expression

The first survey of RAGE expression was conducted shortly after its discovery and demonstrated a low level of transcript expression (as assessed by Northern blot) in all organs excepting the lung, where it was shown to be expressed at very high levels.⁴² There was a notable discrepancy between the mRNA and protein expression profiles, such that not only was the lung not the dominant organ source of RAGE, it was not even first ranked among all the organs assayed. Although the transcript data was later confirmed by other investigators,³⁹ confirmation of the protein data was not readily forthcoming, and over the subsequent years most research has indicated that basal expression of RAGE protein is indeed greatest in the lung.⁴³⁻⁴⁵

The lung is a heterogeneous organ, consisting of many different cell types whose functions range from ensuring adequate ventilation and perfusion to facilitating gas exchange and mediating defense mechanisms against pathogens. The cellular localization of RAGE within the lung was at first somewhat unclear, in part because the discoverers of RAGE and authors of the original survey of its organ distribution suggested that endothelium was the primary source, but also because of an excessive reliance by some on immunohistofluorescence microscopy data (all too often presented without accompanying controls) in the absence of confirmatory biochemical studies. Included among these are studies suggesting that RAGE is expressed by bronchial epithelium,^{46,47} findings that have not been confirmed by the vast majority of investigators. Elegant *in situ* hybridization studies were performed to demonstrate that pulmonary RAGE transcript is localized to type II alveolar epithelial cells⁴⁸; biochemical analysis of type II alveolar epithelial cell primary culture was confirmatory. RAGE transcript localization to type II pneumocytes was not reflected in the numerous protein localization studies that followed, which consistently demonstrated that RAGE protein is expressed by type I

alveolar epithelial cells, the main cell type across which gas exchange occurs.^{39,49-51} Furthermore, a number of these studies^{39,50} incorporated microanatomical dissection by immunoelectron microscopy, which consistently demonstrated that RAGE expression was confined to the basolateral surface of type I pneumocytes. Expression of RAGE transcript primarily by type II pneumocytes is not inconsistent with protein expression primarily by type I pneumocytes, as the latter population arises by differentiation of the former. A unique function of RAGE mRNA apart from its canonical role as a template for protein synthesis is unknown.

Because many diseases, including those of the lung, involve a prominent inflammatory component, it is important to consider RAGE expression in cells of the hematopoietic compartment. Indeed, multiple studies have indicated that cells of the monocyte/macrophage lineage express RAGE,⁵²⁻⁵⁵ although much of this data has been indirect and functional (e.g. demonstration of inhibited uptake of AGE-albumin by monocytes in the presence of anti-RAGE antibodies or co-expressed DN-RAGE). More recently, RAGE has also been linked to T cells,⁵⁶⁻⁵⁸ with some data suggesting a role in T cell activation and cytokine secretion. Limited evidence has been presented demonstrating RAGE expression by eosinophils,⁵⁹ with the suggestion that it may be involved in eosinophil chemotaxis by direct recognition of elaborated RAGE ligands.⁶⁰ Despite the existence of considerable functional and immunocytofluorescence data, apart from one study in human eosinophils⁵⁹ rigorous demonstration of RAGE transcript and protein expression in specific inflammatory cell populations has been lacking.

1.1.3 RAGE in pulmonary disease

Relatively few studies have investigated the relevance of RAGE to pulmonary disease, a fact rendered all the more remarkable given the protein's organ distribution. RAGE has been studied

in cancer, hyperoxic lung injury, sepsis, acute lung injury, chronic obstructive pulmonary disease (COPD), and pulmonary fibrotic disease. RAGE was found to be markedly down-regulated or even totally abrogated in cancerous lung (specifically non-small cell lung cancer, NSCLC), a finding confirmed by multiple groups of investigators.⁶¹⁻⁶⁴ Although the authors infer that this is demonstrative of reduced expression in tumor cells, given the dearth of RAGE in normal bronchus and the proliferative nature of neoplastic disease, it is not unreasonable to attribute decreased global expression either to dysregulated alveolar expression in response to tumor encroachment, or to tumor burden reducing the proportion of RAGE-expressing alveolar epithelial cells in whole lung. Furthermore, while it is unclear whether reduced pulmonary RAGE expression in lung cancer facilitates tumor progression or is merely a bystander effect of disease, the finding that a genetic polymorphism in the RAGE promoter is associated with increased incidence of NSCLC⁶⁵ would tend to support a more mechanistic role.

RAGE has a pernicious pro-inflammatory role in animal models of viral pneumonia, specifically in primary respiratory syncytial virus⁶⁶ and influenza A⁴⁶ infection. The absence of RAGE was found to have a protective effect in mouse models of pneumococcal⁶⁷ and *E. coli*⁶⁸ pneumonia as well as in a cecal ligation and puncture mouse model of sepsis.^{45,69} In contrast, RAGE was found to have a beneficial role in the clearance of mycobacterial lung infection in mice.⁷⁰ Collectively, these studies suggest that pulmonary RAGE has a consistently pro-inflammatory function in the context of bacterial and viral infection.

Limited data is available regarding the role of RAGE in COPD. Soluble RAGE was found to be decreased in lung and in the circulation in patients with COPD with prominent neutrophilic component.⁷¹ Another study demonstrated decreased levels of sRAGE in patients with COPD as compared to controls, with sRAGE levels inversely correlated to emphysema

severity.⁷² Contrastingly, RAGE was also found to be increased in the lungs of COPD patients as compared to those of controls,⁷³ a finding not inconsistent with the sRAGE data. The progressive destruction of lung tissue in COPD may account for the decline in secreted or shed sRAGE, whereas degraded lung architecture and obstructed airflow may drive compensatory up-regulation of RAGE in order to enhance epithelial adhesion to remaining basement membrane.

RAGE's role in pulmonary fibrosis is complex and model-dependent. Mice lacking RAGE were found to develop spontaneous pulmonary fibrosis with age.⁴⁴ RAGE was found to have a deleterious role in bleomycin-induced pulmonary fibrosis in mice.^{74,75} In contrast, RAGE appeared to have no role in silica-induced pulmonary fibrosis,⁷⁶ and in asbestos-induced pulmonary fibrosis the absence of RAGE had an aggravating effect on disease phenotype.^{44,74} In patients with idiopathic pulmonary fibrosis, a disease whose pathogenesis is poorly understood, all RAGE isoforms have been found to be markedly diminished.^{44,77,78} Due to the lack of longitudinal data, it is unclear if loss of RAGE in this disease is causal or consequential. The complex, sometimes seemingly contradictory, roles of RAGE in pulmonary fibrosis in particular and pulmonary disease in general appear to emerge from RAGE's dual function as both a promoter of epithelial adhesion to basement membrane and a pro-inflammatory multiligand receptor.

1.1.4 sRAGE as a therapeutic

As RAGE is thought to be involved as a pro-inflammatory mediator in a variety of disease processes, and sRAGE is known to antagonize ligation of the membrane isoform of RAGE by sequestration of cognate ligands,⁷⁹ sRAGE has been employed as a therapeutic modality in a variety of experimental models of diseases thought to involve RAGE ligands, including

atherosclerosis,^{80,81} wound healing,⁸² ischemia/reperfusion injury,^{83,84} arthritis,⁸⁵ stroke,⁸⁶ and diabetic nephropathy.⁸⁷ Because the vast majority of studies explored sRAGE's therapeutic potential in non-pulmonary disease, it is quite natural that most used intraperitoneal or intravenous modes of delivery. There is reason to suspect that, at least in some cases, purified proteins used in treatment studies may have been contaminated with endotoxin; contaminating endotoxin may have the potential to reroute inflammatory processes away from established inflammatory loci to the site of administration (e.g. the peritoneal cavity), thus simulating a therapeutic effect when in fact there is none. The use of sRAGE synthesized in prokaryotic or simple eukaryotic expression systems, and thus lacking species-appropriate glycosylation, is also a matter of significant concern in evaluating the significance of studies utilizing therapeutic sRAGE.

A graver problem in studies that use sRAGE as a treatment modality is the dearth of pharmacokinetic and pharmacodynamic data. One study in rats using recombinant sRAGE found a plasma half-life of approximately 26 hours when administered intravenously, and a plasma half-life approximately twice as great when sRAGE was administered intraperitoneally.⁸⁸ Appreciable biodistribution to the liver, kidney, and spleen was detected, but no irrelevant protein was used to control for nonspecific effects. Additionally, the large bolus of protein administered (~50-65 µg of sRAGE) may have recruited or induced uptake and clearance mechanisms that are not usually involved basally or in disease states (where circulating sRAGE may be dysregulated). No studies have been conducted with the aim of determining the most efficacious mode of sRAGE delivery to any particular organ, including the lung. Nor has the possibility that sRAGE has roles beyond that of a decoy receptor been explored.

1.2 ASTHMA

1.2.1 Clinical asthma

Asthma is a chronic inflammatory disease of the airways characterized by variable and episodic airflow obstruction, bronchial hyperresponsiveness, and longstanding inflammation that presents with clinical manifestations of wheezing, cough, and dyspnea.⁸⁹ Although both a chronic and obstructive disease, asthma is usually not grouped with COPD, with distinction between the two being made easily on the basis of historic features (e.g. cigarette smoking in COPD) and reversibility. Save in severe cases, airflow obstruction in asthma is largely reversible, whereas in COPD it is minimally so. In addition to reversibility, the existence of characteristic environmental triggers of acute symptoms, such as exercise or exposure to cold air or allergens, is suggestive of asthma. Other historic features that assist in the diagnosis of asthma are early age of onset, a family history positive for allergic diseases, and a history of improvement in symptoms following bronchodilator and corticosteroid therapy.

The non-specificity of symptoms associated with asthma necessitates the use of pulmonary function testing in making a diagnosis.⁹⁰ A series of function tests is usually required. The most commonly employed are the peak expiratory flow rate (PEFR), the forced expiratory volume in 1 second (FEV(1)), and the ratio between FEV(1) and the forced vital capacity (FVC), which relates flow to volume.⁹¹ PEFR is measured during a rapid forceful exhalation immediately following a maximal inhalation; it is most useful in tracking trends in lung function longitudinally.⁹² By itself, a reduction in PEFR is of limited usefulness in making a diagnosis, because it is not specific to asthma or even obstructive lung disease (it is decreased in restrictive lung disease as well), and because it depends largely on patient effort.⁹³ Spirometry provides a

controlled method for assessing airflow and lung volumes and may be utilized to determine numerous indices of pulmonary function. Three are most commonly used in practice: FEV(1), FVC, and the ratio between the two, FEV(1)/FVC. The ratio FEV(1)/FVC is of particular importance in distinguishing between obstructive and restrictive disease, in that while the ratio is reduced in obstructive airway disease, it is commonly normal or even increased in restrictive disease. Measurements of other spirometrically-determined lung volumes and diffusing capacity may be of use in the diagnosis of certain cases of asthma, or in better characterizing physiologic abnormalities in patients already diagnosed.

Due to the episodic nature of symptoms in asthma, two treatment-response tests are often useful in making a diagnosis where the clinical picture, historical features, laboratory test data, and baseline spirometric indices are not definitive. The bronchodilator response test employs a short-acting bronchodilator, such as albuterol, which relieves the reversible airway constriction and airflow obstruction in patients with asthma, which registers as a significant increase in FEV(1) value from baseline. In the event of the patient being already at a true physiologic baseline with minimal constriction at the time of testing, no marked increase in FEV(1) will be detected. Bronchoprovocation testing addresses this possibility: airflow obstruction is induced using a provocative stimulus, such as methacholine. Due to the enhanced bronchial responsiveness in asthmatics, markedly lower doses of methacholine suffice to elicit the same degree of bronchial constriction (as measured by FEV(1) decreased from baseline) in a patient with asthma than a healthy patient or one with non-reversible obstructive disease. Additional tests utilized in the diagnosis or treatment of asthma include assessment of levels of exhaled nitric oxide (which directly correlates with eosinophilic airway inflammation), skin prick testing to determine allergen sensitivity, and chest imaging.⁸⁹

1.2.2 Pathophysiology of asthma

Asthma is a condition characterized by chronic inflammation accompanied by acute symptomatic episodes of reduced airflow that is usually reversible. It is treated as a single disease entity, but in fact consists of multiple subtypes, each with its own distinct pathophysiologic mechanism. However, despite somewhat substantial differences in etiology, triggers, pattern of exacerbation, and responsiveness to therapy, a central common mechanism is thought to be involved, which is the antigen-triggered degranulation of mast cells. Mast cells are innate immune cells resident in the tissues, most frequently in those that interface with the outside environment (i.e. respiratory tract, gastrointestinal tract, etc.)⁹⁴; their primary role is in defense against pathogens. IgE is the key antibody isotype involved in asthma, including both allergic and non-allergic asthma⁹⁵; circulating IgE binds to its high-affinity cognate Fc receptor (FcεRI) on mast cells, thus priming these innate immune cells for specific responses to allergen challenge. Once allergen binds multiple IgE molecules expressed on the surface of mast cells, FcεRI cross-linking takes place and the mast cells degranulate, releasing histamine, serotonin, heparin, eicosanoids, and other inflammatory mediators.^{96,97} Antibody-producing B lymphocytes are induced to shift immunoglobulin production from IgM to IgE by inputs from T lymphocytes in the form of cell-cell contact and soluble cytokines. Interestingly, although intrinsic asthma (which accounts for ~10% of the total number) does not register an identifiable allergen on skin prick test or assays of serum IgE, the pattern of eosinophilic inflammation and expression of IgE at sites of inflammation is remarkably similar to that seen in atopic asthma,⁹⁸ underscoring the centrality of the IgE antibody response and its downstream cellular effectors in asthma. Furthermore, elevated total serum IgE levels were associated with both atopic and intrinsic asthma, although in the latter case the provoking antigen could not be identified.⁹⁵ It is

hypothesized that in intrinsic asthma it is non-classical staphylococcal superantigens (which bypass the usual antigen-IgE antibody-Fc receptor pathway to activate mast cells in a more direct fashion) that drive mast cell degranulation.^{98,99}

Once mast cells degranulate upon encounter of allergen, early and late phase reactions are initiated. The early phase response is mediated by histamine, bradykinin, prostaglandin D2 and leukotrienes LTC4, D4, and E4. The early phase reaction rapidly results in contraction of airway smooth muscle leading to bronchoconstriction,¹⁰⁰⁻¹⁰² which is largely relieved with bronchodilators. The late phase reaction occurs several hours later, involves leukotriene LTB4, and recruits inflammatory cells to the site of allergen insult. During the late phase, bronchoconstriction mediated by airway smooth muscle may also occur but is somewhat less responsive to bronchodilator therapy than bronchoconstriction in the early phase.¹⁰³ The cells recruited during the late phase response include T lymphocytes, dendritic cells, neutrophils, basophils, and eosinophils.

T lymphocytes are obviously crucial in the asthmatic inflammatory response, and are the subject of extensive discussion in a subsequent section. In connection with this, it is notable that non-classical T lymphocytes, including invariant natural killer T cells¹⁰⁴ (iNKTs) and $\gamma\delta$ T cells,¹⁰⁵ are thought to play an important role in asthma pathogenesis. Dendritic cells are professional antigen-presenting cells (APCs) involved in processing allergen and presenting it in a pro-inflammatory context to naïve T lymphocytes, thus activating them. By contrast, resident alveolar macrophages, although professional APCs as well, appear to have an anti-inflammatory effect.^{106,107} Neutrophils have a poorly understood role in asthma; they are primarily associated with more severe phenotypes, which also tend to be more resistant to corticosteroid therapy. As neutrophils are far less susceptible to corticosteroids than eosinophils and many other immune

effector cells,¹⁰⁸ it is possible that severe asthma is often associated with neutrophilia because disease progression and clinical decline occurs more rapidly in the absence of effective corticosteroid inhibition of neutrophils.^{109,110} Basophils are very similar in function to mast cells but may be more important in asthma for their capacity to secrete critical pro-allergic cytokines¹¹¹ than their ability to degranulate as mast cells do. Eosinophils produce effector molecules such as leukotrienes, platelet activating factor (PAF), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and major basic protein (MBP).¹¹² Eosinophils also produce cytokines such as GM-CSF and IL-5, which in turn promote further eosinophil generation and recruitment to the site of inflammation.

The stromal cells of the lung – bronchial and alveolar epithelial cells, smooth muscle cells, fibroblasts, and vascular endothelial cells – are known to play an important role in the pathogenesis of asthma.¹¹³ Epithelium produces eosinophil chemokines RANTES, MIP-1 α , MCP-4, and eotaxin, as well as neutrophil-recruiting IL-8 and pro-fibrogenic TGF- β . In asthma, bronchial epithelial cells also release a variety of growth factors that promote extracellular matrix deposition, including basic fibroblastic growth factor (FGF-2), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), and endothelin-1 (ET-1).¹¹⁴ In asthmatics, airway smooth muscle cells and fibroblasts produce stem cell factor (SCF), which is an important growth factor for mast cells; these structural cells are therefore competent to expand the niche available to mast cells in the lung compartment, thus further promoting the type I hypersensitivity mechanism central to asthma pathogenesis.¹¹⁵ Thus, in the context of asthmatic inflammation, structural cells seem to be involved primarily in secreting chemokines that recruit inflammatory effectors such as mast cells and eosinophils, or in releasing growth factors or enzymes that facilitate remodeling of the airway architecture.

Airway remodeling is a concept that refers to architectural changes in the airways that subsequently superimpose with the underlying inflammation and reversible bronchoconstriction to contribute to irreversible physiologic deficits.¹¹⁶ There are many ways in which this might occur: sloughing of layered epithelium of the bronchus, submucosal gland and goblet cell hypertrophy and hyperplasia leading to excessive mucus occluding the airways, smooth muscle thickening,¹¹⁷ matrix deposition in the subepithelial reticular basement membrane,^{118,119} and aberrant angiogenesis and vascular hypertrophy.¹²⁰ The mechanisms by which each of these forms of airway remodeling lead to functional decline vary: excessive mucus can lead to mucus inspissation and further narrowed or totally occluded airways, whereas deposition of matrix around airways may reduce baseline caliber and thus impede the effect of parenchymal tethering (which maintains airway patency). Regardless of the specific effect on lung structure, airway remodeling occurring over many years leads to progressive decline in measures of lung function both at baseline and during acute bronchoconstrictive episodes.

Airflow obstruction in asthma is primarily reversible and attributable to peribronchial smooth muscle contraction. Bronchoconstriction may be heightened due to intrinsic changes in the smooth muscle cells, neural reflex,¹²¹ or provoking stimuli such as cysteinyl leukotrienes,¹²² prostaglandins, histamine, and tryptase¹²³ released from mast cells, basophils, and eosinophils. Aspirin-induced asthma may also involve these mediators, which then act directly on smooth muscle.¹²⁴ Other less reversible forms of airflow obstruction includes mucus plugging, airway smooth muscle hypertrophy and hyperplasia, and airway edema. Nevertheless, smooth muscle airway constriction constitutes the main source of airflow obstruction. While obstruction occurs throughout the conducting airways, it is the small bronchi that are believed to undergo the bulk of constriction in asthma.¹²⁵ This is not particularly surprising, as physical arguments would

suggest that local airway resistance, the definitive measure of airflow obstruction, is inversely proportional to the radius to the fourth power. Naturally, with equivalent circumferential constriction of smaller and larger bore airways, the smaller bore airways increase in resistance far more rapidly than the larger bore airways. Bronchial hyperresponsiveness arises due to the inflammatory milieu in the pulmonary compartment and is mediated by smooth muscle in response to stimuli such as histamine or methacholine. While it is well known that a number of cytokines are pivotally-involved in bronchial hyperresponsiveness in humans and animal models (discussed subsequently), neither the mechanism by which the smooth muscle hypersensitivity response occurs, nor the potential adaptive effect of that hyperresponsiveness in combatting infection, is known.

1.2.3 Etiopathogenesis of asthma

Asthma is a multifactorial disease thought to arise from complex interactions between endogenous and external factors.⁸⁹ In discussing etiology, it is important to note that asthma consists of many subtypes, e.g. brittle asthma, intrinsic asthma, atopic asthma, aspirin-intolerant asthma, etc.^{126,127} Atopy, which may be understood as the predisposition to develop type I allergic hypersensitivity reactions, is the single most important risk factor in asthma.¹²⁸ Although there is substantial overlap between atopy and asthma, only a fraction of atopic patients develop frank asthma, suggesting that genes involved in asthma risk and asthma severity and those predisposing to atopy are distinct,¹²⁷ a fact attested to by the existence of a distinct population of asthma patients (~10%) who do not demonstrate atopy (intrinsic asthma).¹²⁹ Even so, there is high concordance for asthma in monozygotic twins, indicating that the genetic contribution, although distributed over many loci, is quite strong. The most consistent genetic associations

have been made to polymorphisms in the genes encoding the cytokines IL-4, IL-5, and IL-13.^{130,131} Associations with other genes, such as ADAM-33,¹³² mammalian chitinases,¹³³ and the β_2 adrenergic receptor,¹³⁴ have been made, but the mechanism by which these genes influence asthma pathogenesis is in many cases unclear.

Environmental factors have long been recognized to have a key role in many variants of asthma. One major factor is viral infection.¹³⁵ The role of viral infections in increasing risk of subsequent asthma is complex and depends on the type and site of infection. One study found that RSV bronchiolitis in infancy (that required hospitalization) was associated with early onset asthma.¹³⁶ A second very recent study has demonstrated that risk of later developing asthma was particularly great in children with up-regulated levels of RANTES (an important eosinophil chemokine) in nasal epithelium during RSV bronchiolitis.¹³⁷ This may indicate that RSV bronchiolitis triggers subsequent allergic airway disease in those already predisposed towards eosinophilic inflammatory responses. By contrast, multiple viral infections in early childhood (outside the lower respiratory tract) were associated with decreased risk of developing asthma.¹³⁸ This suggests that whereas early infection of the lower respiratory tract may prime the immune system for subsequent allergic inflammation, the exposure to certain pathogens outside the lung in early life shifts the immune system away from atopic-type inflammation towards a more adaptive immunity.

While it is obvious that allergens are important in triggering symptoms of (atopic) asthma, it is not immediately apparent that they play a significant role in promoting the development of asthma. As it turns out, exposure to certain (primarily indoor) allergens early in life is a major risk factor in the pathogenesis of asthma. In one study, the level of house dust mite and cockroach antigen to which children were exposed was directly related to sensitization

risk.¹³⁹ Exposure to higher levels of cockroach allergen was shown to be associated with greater risk of sensitization and subsequent development of asthma in a study in inner-city children.¹⁴⁰ Other investigators found that exposure to one of the main house dust mite allergens, Der p 1, was demonstrated to be a determinant of the risk of subsequent asthma development.¹⁴¹ Even exposure to low levels of allergen is sufficient to increase asthma risk: sensitization to the outdoor fungus *Alternaria* was found to be an important independent risk factor for asthma in children.¹⁴² Collectively, these data suggest that increased exposure to certain allergens (with presumably intrinsic pro-allergenic characteristics) increases risk of sensitization; as many subtypes of asthma are marked by prominent atopy, increased risk of sensitization implies an increased risk of subsequent development of asthma as well. Other important environmental factors thought to contribute to asthma pathogenesis include exposure to tobacco smoke^{143,144} and air pollution,¹⁴⁵ vitamin D deficiency¹⁴⁶ and a diet low in omega-3 fatty acids,¹⁴⁷ exposure to organic solvents,¹⁴⁸ and maternal use of paracetamol during pregnancy.¹⁴⁹

1.2.4 Allergic mechanisms of asthma

That allergic inflammation is central to the pathogenesis of atopic asthma was first appreciated from autopsy specimens from patients with fatal asthma.¹²³ Biopsies from patients with less severe disease confirmed this finding, with the degree of inflammation often directly correlated with severity of disease.^{150,151} Since then, these observations and those gleaned from other diseases encouraged research into the mechanisms of innate and adaptive immunity mediating allergy. Immunologists had long recognized that the inflammatory processes involved in acute infection and allergic hypersensitivity are markedly different: whereas acute bacterial and viral infections involve predominantly neutrophilic, lymphocytic, and monocytic inflammation with

associated opsonizing IgG and IgA antibodies and cytotoxic lymphocytes, in allergic hypersensitivity reactions it is degranulating mast cells, infiltrating eosinophils, and non-opsonizing IgE antibodies that are most prominent. Interestingly, the inflammatory pattern in allergic disease is remarkably similar to that observed in parasite and helminth infections,¹⁵²⁻¹⁵⁴ suggesting that this type of inflammation – so pernicious in response to innocuous allergens – is in fact adaptive in combating infection by large extracellular pathogens.

Biologists seeking to understand the role of T cells in the immune response (whose importance in antigen recognition and the host response was then only beginning to be fully appreciated with the discovery of MHC restriction¹⁵⁵) discovered that non-cytotoxic (helper) T cell clones from mice demonstrated two major patterns of cytokine secretion.¹⁵⁶ They named these two types of helper T cell responses T-helper 1 (Th1) and T helper 2 (Th2). Th1 has since come to be recognized as mediating adaptive immune responses to intracellular pathogens as well as certain kinds of autoimmune diseases, with a secretory profile notable for IFN- γ and TNF- β and effectors such as cytotoxic T cells, activated macrophages and opsonizing IgG antibodies.¹⁵⁷ Th2 is thought to mediate responses to extracellular pathogens such as parasites and helminths as well as atopic disease, via effectors such as IgE, eosinophils, basophils and mast cells, and changes in tissue architecture (e.g. mucus hypersecretion) believed to facilitate expulsion of such pathogens.¹⁵⁷ Much of the work that contributed to this understanding of fundamental T cell biology was initially performed in mice. The finding that polymorphisms in IL-4,¹⁵⁸ IL-5,¹⁵⁹ and IL-13¹⁶⁰ or their cognate receptors are linked to human asthma therefore lent support to the thesis that atopic asthma is mediated primarily by a Th2 process. Further confirmation was provided by the observation that these cytokines are expressed at sites of inflammation in the lungs of patients with allergic asthma.¹⁶¹⁻¹⁶³

The finding that Th1 and Th2 immune responses appear to be reciprocally-inhibitory of each other,¹⁶⁴ as well as the observation that asthma prevalence is drastically increasing in the developed world¹⁶⁵ (where childhood incidence and severity of infection has declined) promoted the formulation of the so-called ‘hygiene hypothesis.’^{166,167} This hypothesis holds that humans are skewed towards Th2-type responses early in life. Encounters with Th1-inducing pathogens, such as bacteria and viruses, redirect the baseline immune response towards a more Th1-type phenotype, thus reducing the risk of Th2-mediated diseases, such as atopic asthma. As more developed countries have improved sanitation, better patient education, and better access to antibiotics, the incidence and severity of infections that elicit a Th1 response has markedly declined, thus maintaining more individuals in a Th2-skewed state, with a greater predisposition towards developing asthma. There is some direct support for the hypothesis in the form of reduced risk of asthma in those who have experienced infections with hepatitis A virus¹⁶⁸ or *Mycobacterium tuberculosis*.¹⁶⁹ The hypothesis also potentially explains the association between daycare attendance, later birth order and larger family size with decreased incidence of asthma.^{170,171} Although intellectually compelling, the hygiene hypothesis fails to explain the increased incidence of Th1-mediated autoimmune diseases, such as type 1 diabetes mellitus¹⁷² and multiple sclerosis,¹⁷³ in the industrialized world,¹⁷⁴ nor the fact that helminth and parasite infection – rather than stabilizing an innate Th2 bias and thereby increasing predisposition to atopic asthma, as would be expected – appears to be negatively associated with subsequent development of atopy and asthma.¹⁷⁵

The Th2 immune response is coordinated by a canonical triad of cytokines: IL-4, IL-5, and IL-13. IL-4 appears to have a crucial role in the sensitization process that primes naïve cells to differentiate into Th2 cells: loss of IL-4 at the gene level leads to attenuation of allergic

inflammation,¹⁷⁶ while administration of antibodies against IL-4, but not IL-5, during the sensitization process leads to impeded differentiation to Th2 and secretion of downstream cytokines.^{177,178} However, inflammation is intact when IL-4 was antagonized during the challenge phase, after sensitization has taken place.^{177,178} This indicates that IL-4 is involved in the early part of Th2 inflammation, but is not necessary for continued maintenance of allergic inflammation once T cells have been primed.¹⁷⁹⁻¹⁸¹ IL-4 is also crucial for the generation of an IgE antibody response.¹⁸²⁻¹⁸⁶ IL-4 is necessary for priming and activation of T cells to express the co-stimulatory molecule CD154; subsequent IL-4 ligation of its cognate receptor on B cells, as well as T cell CD154 ligation of B cell CD40, induces B cells to undergo antibody isotype class switching from IgM to IgE and differentiation to long-lasting plasma cells. The source of early IL-4 was for some time a mystery, as IL-4 is necessary for Th2 differentiation, but non-Th2 cell sources of this cytokine were unknown. It appears that early IL-4 is derived from innate immune cells, such as basophils, which are increasingly being recognized as key players in the initiation of the allergic immune response.¹⁸⁷⁻¹⁸⁹

The second member of the classic Th2 cytokine triad, IL-5 is the cytokine principally responsible for eosinophil generation, differentiation, maturation, recruitment, survival, and activation. IL-5 is also involved in terminal differentiation of B cells to IgE-producing plasma cells.^{190,191} Administration of antibodies against IL-5 markedly reduces the eosinophilic component of inflammation in animal models of asthma.¹⁹² Furthermore, mice lacking IL-5 were found to have abolished eosinophilia, airway damage, and airway hyperreactivity.¹⁹³ Conversely, overexpression of IL-5 in airway epithelium¹⁹⁴ or administration of exogenous IL-5¹⁹⁵ were found to induce pronounced tissue eosinophilia, suggesting that IL-5 is both necessary and sufficient for eosinophil recruitment. IL-5 has been long considered to be primarily produced by

Th2 cells: IL-4 ligation of its cognate receptor on T cells activates STAT6 and induces expression of the GATA-3 transcription factor, which is the central regulator of Th2 differentiation and coordinates expression of IL-4, IL-5, and IL-13.^{196,197} This simplistic view is undergoing revision, as it is increasingly recognized that many innate effector cells produce IL-5 as well, including eosinophils themselves,¹⁹⁸ mast cells,¹⁹⁹ and a novel class of innate lymphocytes called nuocytes.²⁰⁰ The transcriptional regulation of IL-5 production in these cell types is not well understood.

Eosinophil recruitment is mediated by a diverse group of chemokines, including but not limited to RANTES (CCL5), MCP-3 (CCL7), MCP-4 (CCL13), MIP-1 α (CCL3), TARC (CCL17), eotaxin (CCL11), and eotaxin-2 (CCL24).²⁰¹ Of the aforementioned, however, only eotaxin and eotaxin-2 (and eotaxin-3 (CCL26), in humans) exhibit eosinophil-specific chemotactic activity through the chemokine receptor CCR3. Indeed, eotaxin has no effect on neutrophils and macrophages, which do not express CCR3,^{202,203} and only a weak effect on conditioned lymphocytes.²⁰⁴ Eotaxin-2 is chemotactic for lymphocytes, but only weakly-so for neutrophils.²⁰⁵ Eotaxins are synthesized and secreted by structural cells of the lung, including fibroblasts^{206,207} and smooth muscle cells.²⁰⁸⁻²¹⁰

Transmigration of eosinophils into the interstitium and peribronchial and perivascular spaces requires the expression of adhesion molecules mediating eosinophil extravasation. One of the key endothelial binding partners of the eosinophil-expressed integrin VLA-4 (expressed on many cell types, but apparently the dominant integrin expressed on eosinophils)²¹¹ is vascular cell adhesion molecule 1 (VCAM-1).²¹²⁻²¹⁴ Absence of VCAM-1 seriously impairs eosinophil migration into the lung in response to allergic stimulus²¹⁵ and Th2 cytokines associated with asthma, in turn, drive up-regulated expression of VCAM-1.^{216,217} Another key endothelial

binding partner of eosinophils is P-selectin, which interacts with eosinophil P-selectin glycoprotein ligand 1 (PSGL-1).²¹¹ While both eosinophils and neutrophils express PSGL-1, eosinophils demonstrate enhanced efficiency of initial binding to P-selectin and thus are selectively recruited.²¹⁸ Interestingly, platelet-expressed P-selectin also mediates eosinophil recruitment through the formation of transient platelet-eosinophil circulating aggregates that promote adhesion to vessel walls and subsequent transmigration.²¹⁹

IL-13 is the third member of the set of canonical Th2 cytokines. It acts in a complementary fashion to IL-5 by inducing expression of the eotaxins.^{206,220-222} Furthermore, the receptor for IL-13 shares its IL-4R α subunit with the IL-4 receptor, which leads to a degree of overlap in the actions of the two cytokines. For instance, IL-13 is necessary for basal IgE production, but does not play the decisive role in inducing class switching (which is mediated by IL-4). IL-13 has been recognized as having a vital role in the genesis of airway hyperreactivity.²²³ This was demonstrated through global deletion of IL-13 abrogating airway hyperresponsiveness, despite the presence of eosinophilic inflammation; supplementation of exogenous IL-13 to knockout animals restored airway hyperresponsiveness. These findings were previously seen with antibody-mediated antagonism of IL-13.²²⁴ IL-13 is also believed to drive aspects of airway remodeling, such as subepithelial fibrosis,²²⁵ which may be attributable to IL-13 induction of TGF- β synthesis by stromal cells.²²⁶ The role of IL-13 in promoting mucus hypersecretion in allergic inflammation is critical.^{224,225,227} Th2 cells in the absence of IL-13 have been shown to be incapable of inducing mucus secretion in mouse models of asthma; furthermore, IL-9's effect on mucus secretion has been found to be mediated via IL-13.²²⁸ Inhibition of IL-13 using antagonistic antibodies was found to markedly attenuate mucus secretion, whereas administration of exogenous IL-13 was sufficient to induce it.²²⁹ Collectively,

these observations suggest that just as IL-4 and IL-5 have unique roles in mediating isotype class switching and eosinophil recruitment, respectively, IL-13 is the main Th2 cytokine involved in tissue changes in allergic inflammation (the adaptive role is presumably to condition the respiratory and gastrointestinal tracts for easier expulsion of parasites secondary to eosinophil attack and mast cell degranulation). IL-13 was initially thought to be produced primarily or entirely by Th2 cells, but it has since been appreciated that innate cells called nuocytes also produce the cytokine in a STAT6/GATA-3-dependent manner.²³⁰

Initially grouped with the Th2 cytokines, IL-9 demonstrates a complex expression pattern, with some secretion by Th2 cells but most expression being driven by a separate differentiated class of T lymphocytes, Th9 cells.¹⁵⁷ IL-9 overexpression in pulmonary epithelium in mice leads to mucus hypersecretion and eosinophilia, airway hyperresponsiveness to methacholine challenge, and subepithelial fibrosis. IL-9 overexpression is also uniquely accompanied by prominent mast cell infiltrates.²³¹ IL-9 over-expressed in pulmonary epithelium apparently mediates its role through classic Th2 cytokines, up-regulating their expression; conversely, blockade of the downstream cytokines IL-4, IL-5, and IL-13 attenuates or completely abolishes the effects of IL-9 on eosinophilia and mucus production.²³² However, deletion of IL-9 does not abrogate allergic inflammation, with IgE, IL-4, IL-5, and IL-13 intact, along with their usual effects on mucus production and eosinophilic inflammation.²³³ This suggests that IL-9 is not as fundamental to Th2 immunity as the classic triad and that it acts more as an ancillary cytokine of allergic inflammation, with the capacity to drive inflammation but not central to it.

Although not a classic cytokine of the Th2 immune response, IL-17 has been suggested to be a molecular mediator relevant to asthma, particularly in subtypes with a prominent

neutrophilic component.^{234,235} While IL-17 has been shown to play a role in neutrophilic asthma,²³⁶ it has also been shown to be a negative regulator of established allergic asthma.^{237,238} Furthermore, the level of IL-17 expression in asthmatics has been shown to associate with bronchial hyperresponsiveness.²³⁹ A number of mouse models of allergic airway disease/asthma, including T cell adoptive transfer models, have recapitulated this observation, with IL-17 inhibition effected with antagonistic antibodies leading to attenuation in airway hyperresponsiveness.^{240,241} IL-17 is of particular interest for its potential role in steroid-resistant asthma²⁴² and severe asthma.¹⁰⁹

1.2.5 Initiation of allergy

Although the last two decades of research have elucidated much about the mechanisms underlying allergic inflammation downstream of the T cell, the factors initiating Th2 immune responses remain poorly understood. Recently, a set of innate immune cytokines involved in initiation of Th2 inflammation has been identified, and their potential role in atopic asthma is a matter of great interest. The first of these, thymic stromal lymphopoietin (TSLP), is synthesized and secreted by epithelium,²⁴³ basophils, and mast cells²⁴⁴ in response to activation by a protease receptor, PAR-2.²⁴⁵ Activation of the receptor is mediated by protease activity, which is a common feature in allergens, including house dust mite and fungus. This places TSLP very early in the immune response at the level of allergen recognition, considerably upstream of T cell differentiation. Antibody-mediated inhibition of TSLP or deletion of its cognate receptor (TSLPR) attenuates allergic inflammation.²⁴⁶ The observations from animal models and *in vitro* studies attesting to the importance of TSLP are supported by the finding that polymorphisms in

this gene are linked to susceptibility to bronchial asthma in humans.²⁴⁷ TSLP is thought to be important for the synthesis and secretion of IL-4, IL-5, IL-13, IL-10, and IgE.^{248,249}

IL-25 (IL-17E) is gaining increasing prominence as a potent stroma-derived Th2-promoting cytokine. IL-25 is structurally related to IL-17, but in contrast to the latter, which is primarily secreted by T lymphocytes and coordinates the adaptive immune response and its neutrophil effectors, IL-25 is released by structural cells (specifically pulmonary epithelium) and induces pathologic changes and cytokine profiles consistent with Th2 immunity.²⁵⁰⁻²⁵² Moreover, IL-25 exerts its effects in an IL-4- and GATA-3-dependent manner in T cells,²⁵³ but most of the IL-5 and IL-13 generated in response to IL-25 originates from innate lymphocytes called nuocytes.²⁵⁴ Administration of exogenous IL-25 induces expression of IL-4, IL-5, and IL-13 in the absence of allergen.²⁵⁰ Conversely, inhibition of IL-25 action through the use of blocking antibodies diminishes the Th2 response and airway hyperresponsiveness in models of allergic asthma, even when blockade is initiated after sensitization has already occurred (indicating effects beyond a role in the initiation of Th2 differentiation).²⁵⁵

The third member of the recently-identified group of stroma-derived pre-Th2 cytokines, IL-33 is constitutively expressed in endothelium, lung, colon, and brain, among other tissues.²⁵⁶ IL-33 is structurally related to IL-1 and IL-18, but does not require cleavage by caspase-1 to be activated.^{257,258} It has a dual function: normally it acts as a negative regulator of transcription, localizing primarily to the nucleus and associating with heterochromatin.²⁵⁹ In response to insults, however, IL-33 is released either passively due to loss of integrity of the nuclear envelope and cell membrane or in a regulated fashion. In this sense, it appears to act as an alarmin,²⁵⁶ analogous to HMGB1 and IL-1 α . Endogenously-released IL-33 can bind to its cognate receptor (ST2) on inflammatory cells to induce a Th2-type response.^{260,261} IL-33 is able

to do so even in SCID mice (which lack an adaptive T cell response) suggesting that it is able to exert its cytokine-inducing function purely through innate immune cell and structural cell targets.²⁶² Lack of IL-33 leads to markedly attenuated Th2 immune responses to allergen, indicating that it is critical in the development of a competent allergic response.²⁶³ Furthermore, exogenous IL-33 drives induction of Th2 cytokines, indicating that it is a central mediator in a common pathway leading to allergic inflammation and does not depend on PAMPs or DAMPs for activity. IL-33-induced IL-5 and IL-13 are primarily secreted by innate lymphocytes called nuocytes.²⁵⁴

Nuocytes have been recently identified as having a crucial role in the development of Th2 inflammation in animal models of pulmonary and gastrointestinal disease.²⁶⁴ Although they do not bear lineage markers of T cells or B cells, they bear CD45 and the IL-7 receptor, which suggests a lymphoid origin. In addition to these markers, nuocytes bear the receptors for IL-10, IL-12, and IL-17, as well as IL-25 and IL-33 (ST2). IL-33 together with IL-7 has the capacity to induce nuocyte proliferation, whereas IL-25 and IL-33 can independently induce secretion of IL-5 and IL-13. Nuocytes were shown to have the capacity to induce IL-13-mediated expulsion of gastrointestinal nematodes in the absence of T cells; by contrast, competent Th2 cells alone, in the absence of nuocytes, were not able to clear the parasites.²⁵⁴ In another study in mice with global deletion of IL-13 (and thus resistant to the development of airway hyperresponsiveness and inflammation more generally), adoptive transfer of nuocytes expressing IL-13 could restore susceptibility to IL-25-induced bronchial hyperreactivity and pulmonary inflammation.²⁶⁵ This suggests that nuocytes alone produce sufficient quantities of IL-13 to reconstitute the entire IL-13 axis in models of Th2-mediated disease. In connection with this, in a mouse model of helminth infection it was found that all nuocytes express IL-13, 70% express IL-5, and less than

5% express IL-4.²⁵⁴ This would suggest that nuocytes are involved in coordinating innate immune cell and structural cell responses without significantly driving T cell differentiation to Th2, this latter function presumably being relegated to IL-4-producing basophils.

This discussion of allergic mechanisms of asthma is summarized schematically below (Figure 1).

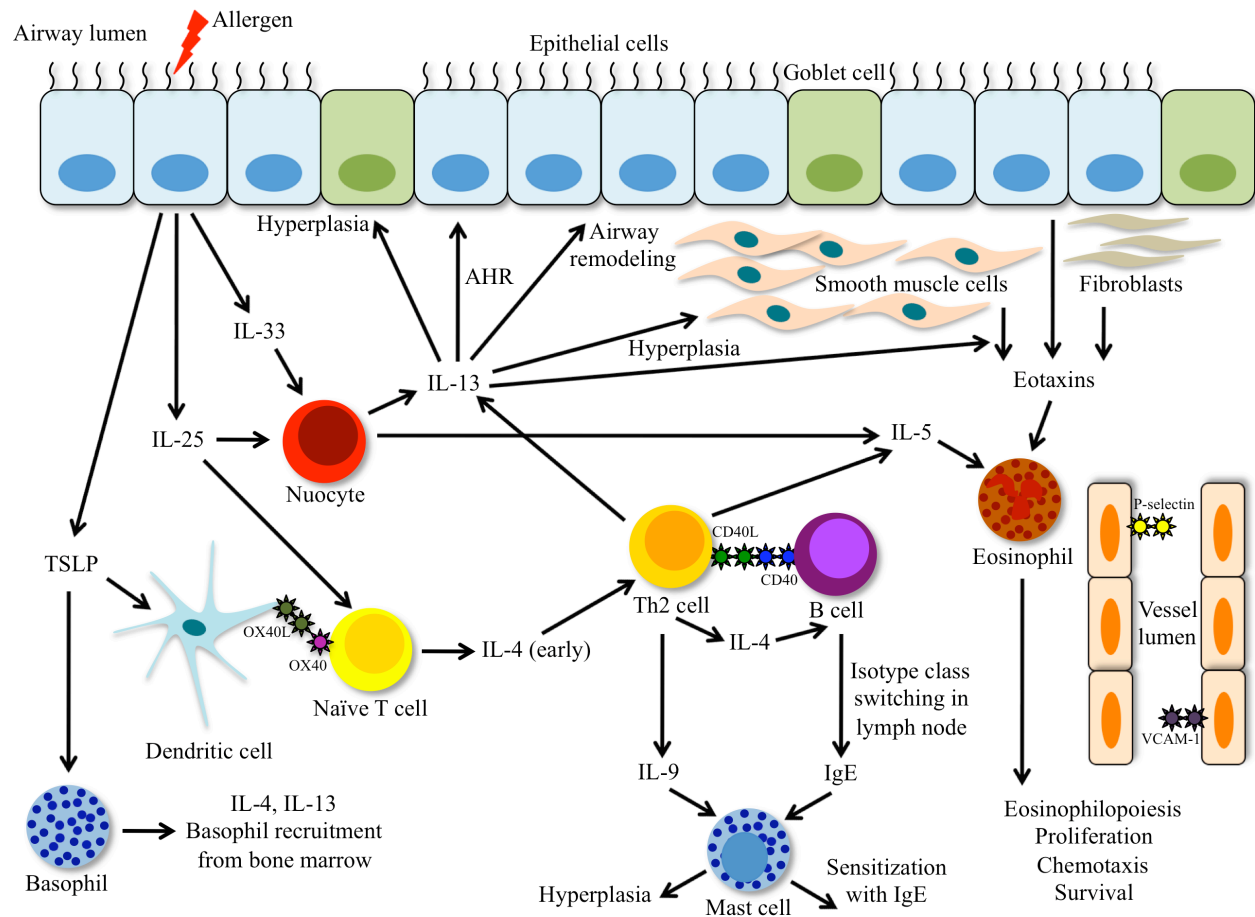


Figure 1. A schematic representation depicting major pathways of Th2 immunity.

Allergen along with danger signals elicits secretion or release of TSLP, IL-25, and IL-33. TSLP induces IL-4 from basophils and T cells. IL-25 induces IL-4, IL-5, and IL-13 in T cells and nuocytes. IL-33 induces IL-5 and IL-13 in nuocytes. IL-4 drives Th2 differentiation and isotype class switching in B cells. IL-5 drives eosinophil generation, proliferation, survival, and recruitment (also mediated by eotaxins). IL-9 promotes mast cell hyperplasia and mucus hypersecretion. IL-13 induces airway hyperreactivity (AHR), airway remodeling and smooth muscle hyperplasia, goblet cell hyperplasia and mucus hypersecretion, eotaxin expression, and up-regulation of adhesion molecules in vasculature to promote eosinophil transmigration.

1.2.6 Asthma therapy

Asthma treatment must address both the underlying pathogenic process driving the disease, as well as the episodic symptoms arising from acute constriction of the airways. The mainstay of asthma therapy is long-term suppression of inflammatory airway disease with low- or medium-dose inhaled corticosteroids. Short-acting aerosolized β_2 -agonists, which target airway smooth muscle, are used to control bronchoconstriction.¹²³ In patients with more severe asthma, the dose of inhaled corticosteroids must be ramped up, short-acting bronchodilators may need to be exchanged for longer-acting agonists, and occasionally oral corticosteroids may be introduced as well.²⁶⁶ In patients that do not tolerate steroids well or have disease refractory to these conventional treatment regimens, alternative therapies must be considered. Steroids are quite effective in most patients precisely because they target so many genes and cell types involved in inflammation.^{267,268} Therefore, when a maximal steroid dose has been found to be inadequately effective, agents targeting specific molecular or cellular players in asthma are used. These have conventionally included the cromoglycates, which are thought to target mast cell function, as well as the leukotriene modulators.²⁶⁷ The cromoglycates have declined in popularity due to no added benefit beyond inhaled corticosteroids, whereas the leukotriene modifiers have become the main non-steroidal therapy for control of inflammation in asthma. These drugs block leukotriene access to the cognate type 1 cysteinyl leukotriene receptor.²⁶⁹ Leukotriene modulators may be most effective in mild persistent asthma as an alternative to corticosteroids, and may offer some additional benefit in patients with severe asthma already treated with high-dose corticosteroids,²⁷⁰ but the combination of corticosteroids and long-acting bronchodilators remains superior.²⁷¹

Although a heterogeneous disorder, several central molecular mediators of asthma have been identified, and targeting them via small molecule inhibitors or antagonistic antibodies would appear to be a viable approach in the treatment of asthma in which steroids, bronchodilators, and leukotriene modifiers are inadequate. Anti-IgE therapy (omalizumab) is the first of these biological immunoregulatory agents to have entered the mainstream, and has been shown to reduce the frequency of exacerbations in severe allergic asthmatics.^{272,273} Anti-IL-5 therapy (mepolizumab) has also had modest success in reducing exacerbation frequency and permitting steroid dose reduction in patients with eosinophilic asthma.^{274,275} Antagonism of the IL-4 receptor α chain (which is part of both the IL-4 receptor and IL-13 receptor) failed to demonstrate clinical efficacy in asthmatics²⁷⁶; interestingly, inhibition of solely the IL-13 signaling pathway with an antibody against the cytokine (lebrikizumab) demonstrates very modest but statistically significant improvement in lung function in patients with asthma, particularly those with high IL-13 activity.²⁷⁷

The results seen in the use of biological agents against cytokines, their receptors, and cellular effectors (despite the eminent rationality in their being targeted) have been less than satisfactory, as these agents are often accompanied by exorbitant cost in exchange for modest benefit, risk of additional morbidities, and are confined in their effectiveness to narrow subsets of asthmatics (e.g. asthma with prominent atopic component for omalizumab and eosinophilic asthma for mepolizumab). Redundancy in the system may render targeting of any specific downstream effector cell type or terminal cytokine relatively ineffective at ameliorating disease in the majority of patients. Identification of fundamental nodes higher up in the molecular hierarchy coordinating the pathogenesis of allergy and asthma may be a prerequisite for the development of the next generation of broadly effective therapeutics.

1.3 RAGE IN ASTHMA

1.3.1 Genome-wide association studies

Genome-wide association studies (GWAS) are epidemiologic analyses that link common genetic variants, most often single-nucleotide polymorphisms (SNPs), to traits such as disease status, biomarker level, or a physiologic measure. As such, they are powerful tools in the assembly of working hypotheses that would not have been otherwise apparent based on current knowledge; these hypotheses can then be explored in depth in humans or animal models. Two recent GWAS analyses were performed,^{278,279} studying genetic loci linked to the spirometric parameter FEV(1) and the FEV(1)/FVC ratio. Reduced FEV(1)/FVC ratio is indicative of obstructed airflow, whereas FEV(1) serves as an indicator of severity of obstruction and may be used longitudinally to track progression of disease.²⁸⁰ Both meta-analyses were conducted on two different consortia of European patients. The first group,²⁷⁸ working on the SpiroMeta consortium, identified six loci linked to reduced FEV(1) or FEV(1)/FVC, whereas the second group,²⁷⁹ working on the CHARGE consortium, identified eight loci linked to reduced FEV(1)/FVC ratio and one to FEV(1). In both studies, the association between reduced FEV(1)/FVC and *RAGE* (termed *AGER* in their studies) was strong. *RAGE* was a notable finding in both studies, in that the first study²⁷⁸ stated that it was the strongest association to FEV(1)/FVC of all the loci identified, whereas the other study²⁷⁹ pointed out that a *RAGE* SNP was one of a small group of three (out of nearly 120 in total) SNPs that was localized to the exonic region of the implicated genes and thus could exert its effects through altered protein structure.

That SNP, rs2070600, is a glycine-to-serine missense mutation at the codon for residue 82 (G82S), which places the mutation precisely at the second of two N-glycosylation sites

(asparagine at position 81).⁸ As previously discussed, glycosylation plays an important role in RAGE function, and a mutation introducing bulkier and hydrophilic serine in place of glycine might be expected to interfere with that post-translational modification and thus impede RAGE function. In fact, the contrary appears to be the case. It was recently shown that RAGE bearing the G82S polymorphism expressed in HEK 293 cells, in comparison to wild type RAGE expressed in the same system, demonstrates preferential glycosylation of the asparagine at residue 81.²⁸¹ Using the same *in vitro* system, the authors also demonstrated enhanced nuclear translocation of NF- κ B in response to S100B stimulus in HEK 293 cells expressing RAGE bearing the rs2070600 SNP as compared to those transfected with wild type RAGE, confirming the results of an earlier study exploring the same polymorphism's role on RAGE function.²⁸² In the event that these *in vitro* observations hold true in the lung, it would appear that enhanced RAGE function correlates with increased risk of obstructive lung disease.

1.3.2 Clinical studies

A very few preliminary studies have explored the expression of RAGE or RAGE ligands in patients with asthma. S100A12, a RAGE ligand, was recently shown to be expressed in eosinophils and up-regulated in sputum from patients with eosinophilic asthma (as compared to patients with other asthma subtypes and normal controls).²⁸³ HMGB1 was shown by one group to be up-regulated in induced sputum from patients with asthma in general and those with neutrophilic asthma in particular, a finding confirmed by other investigators.²⁸⁴ The same group demonstrated significantly higher levels of esRAGE in asthmatics, but no correlation between esRAGE level and disease severity.²⁸⁵ However, another group studying sRAGE and HMGB1 in asthma found that while sRAGE was unchanged from the normal baseline in most asthma

subtypes, it was decreased in those with neutrophilic asthma, a phenomenon they attributed to increased degradation by neutrophils.⁷¹ This group also found no difference in HMGB1 expression between asthmatics (regardless of subtype) and healthy controls. No attempt by the latter group to reconcile their data with the work of others was made. Such inconsistency in a very small body of literature is worrisome; moreover, these studies have failed to provide mechanistic insight as to the role, if any, of RAGE or RAGE ligands in asthma.

1.3.3 sRAGE-induced pulmonary inflammation

Previous work has suggested that exogenous sRAGE delivered to the lung may induce monocyte and neutrophil²⁸⁶ or eosinophil³⁸ ingress. In the first study, a bolus of up to 100 µg of recombinant baculovirus-expressed sRAGE, ostensibly depleted of endotoxin, was administered intratracheally to individual mice. One or two days later, lungs were harvested and examined, demonstrating an influx of monocytes and neutrophils into the pulmonary compartment. The authors apparently infer that the fact that purified sRAGE was more effective than endotoxin at recruiting monocytes and less effective than endotoxin at recruiting neutrophils was evidence that contaminating endotoxin was not responsible for the phenomena seen.²⁸⁶ Another study³⁸ suggested that bovine sRAGE administered intratracheally (as a therapeutic in a bleomycin model of pulmonary fibrosis) had the capacity to recruit eosinophils to the lung, even in control mice that were not receiving the chemotherapeutic agent. At first mistaking eosinophils for neutrophils, the investigators repeatedly endotoxin-depleted their preparations of sRAGE, believing it to be the cause of the unanticipated granulocytic inflammation they were seeing. It is notable that two instillations of sRAGE to the lungs, spaced out over several days, were markedly more effective at inducing eosinophil chemotaxis than a single instillation.

2.0 RATIONALE AND HYPOTHESIS

The receptor for advanced glycation end products (RAGE) was first identified as a potential pro-inflammatory mediatory of vascular disease in diabetes.¹ The putative role in diabetes and inflammation, as well as the various ligand identifications made, had the effect of steering RAGE biology research away from questions of homeostasis and almost exclusively towards those of pathogenesis. Curiously enough, the fact that RAGE is expressed in the lung (and in the structural cells of the lung, at that) at levels overwhelmingly greater than in any other organ appeared for many to be a very distant consideration. Although hypotheses referential to purposes rather than means or mechanisms are no longer acceptable in mainstream scientific discourse, such a specific distribution is undeniably compelling and mandates further investigation.

RAGE's capacity as a pro-inflammatory multiligand receptor suggests that it may be involved in the recognition of pathogens. Distribution across broad surfaces of alveolar epithelium would lend RAGE the capacity to sense large-scale pathogen motifs, such as the repeat motifs found in parasite glycocalyx and helminth tegument; this is a particularly interesting possibility, as the immune response to parasites and helminths appears to be related in a fundamental way to atopic diseases such as asthma. Remarkably, however, no investigators have published studies of RAGE in animal models of allergic airway disease/asthma.

Recent whole genome association studies have demonstrated a strong correlation between a decline in the ratio FEV(1)/FVC and the RAGE gene.^{278,279} Although such an association is not specific with respect to the type of obstructive disease, asthma is certainly consistent with such a change in lung function. In connection with this, it would appear that at this time there are only two studies dealing directly with RAGE in patients with asthma^{71,285} (additional studies address RAGE ligands S100A12²⁸³ and HMGB1²⁸⁴). The findings of the two studies are mutually contradictory, primarily concerned with the neutrophilic subtype of asthma, and provide no mechanistic insight into the role of RAGE and specifically sRAGE in the disease. A better understanding of the potential role of RAGE in allergic airway disease/asthma requires more invasive studies in humans or at the very least the use of animal models, where genetic heterogeneity and environmental non-uniformity are minimized, genetic engineering techniques are available, and pharmacologic agents may be easily tested.

The arguments and observations described above led to the formulation of the hypothesis that *pulmonary RAGE is an important mediator of allergic airway disease/asthma and impeding its effect, either through complete elimination of the encoding gene or via pharmacologic blockade, attenuates or abolishes one or more key features of the asthmatic phenotype.*

3.0 MATERIALS AND METHODS

3.1 ANIMAL STUDIES

3.1.1 Animals

Wild type male C57BL/6 mice were purchased from Taconic or Charles River. Founder RAGE knockout (RAGE KO) mice were provided by Dr. A. Bierhaus (University of Heidelberg) and from these a breeding colony was initiated.^{44,76,287} These mice are congenic with the C57BL/6 background. RAGE KO mice were age- and sex-matched to wild type mice for each experiment. In all cases mice were housed in the animal care facility of the University of Pittsburgh and experimental protocols were approved by the University's Institutional Animal Care and Use Committee.

3.1.2 Protein preparations

Soluble RAGE (sRAGE) was purified from mouse lung tissue and endotoxin was removed with a Detoxi-Gel column (Pierce) as described previously.^{7,37} Purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining. Specific binding of the purified sRAGE to a known RAGE ligand, HMGB1, was assessed as described elsewhere³⁹ to ensure ligand binding was intact in the purified protein.

Chromatographically purified mouse serum albumin (MSA) and ovalbumin (OVA) were purchased from Sigma. House dust mite (HDM) extract was obtained from Greer Laboratories. HMGB1 was kindly provided by Dr. K. Tracey (Feinstein Institute for Medical Research).

3.1.3 Radiolabeling of proteins

Purified mouse serum albumin and mouse sRAGE were labeled with Na¹²⁵I (Perkin Elmer) using the chloramine T method (Iodination Beads, Pierce) per manufacturer's instructions. To remove residual iodine, both samples were run over Bio-Spin 6 (Bio-Rad) centrifugation columns and the eluate was collected. Protein concentration was determined using the absorbance at a wavelength of 280 nm, the experimentally-determined mass extinction coefficient of mouse sRAGE of $1.89 \times 10^{-3} \text{ mL } \mu\text{g}^{-1} \text{ cm}^{-1}$, and the commonly reported mass extinction coefficient of serum albumin of $0.52 \times 10^{-3} \text{ mL } \mu\text{g}^{-1} \text{ cm}^{-1}$. Gamma counting was performed on a Cobra II auto gamma counter (Packard, Perkin-Elmer) and activity calculated from disintegrations per minute and the apparatus' counting efficiency. Samples were analyzed by SDS-PAGE and gel autoradiography to estimate purity.

3.1.4 Delivery of radiolabeled proteins

Eight-week-old male wild type mice were administered radioiodinated MSA or mouse sRAGE via intratracheal (i.t.), intraperitoneal (i.p.), or intravenous (i.v.) routes. Each animal received 1 μCi of radiolabeled protein, which corresponded to $\sim 0.6\text{-}0.9 \text{ } \mu\text{g}$ of mouse serum albumin or $\sim 0.7\text{-}1.4 \text{ } \mu\text{g}$ of mouse sRAGE (the range is due to the time over which the experiments were

performed and the radioactive decay of the ^{125}I isotope), diluted in 0.9% saline; for i.t. and i.p./i.v. treatments, the treatment volumes were 70 μL and 200 μL , respectively.

3.1.5 Processing of animals following radiolabeled protein biodistribution

Mice were sacrificed at 1, 2, 4, and 12 hours after the administration of radiolabeled protein, and 4-5 mice were used per treatment group (where a treatment group was defined by administration route, protein administered, and time point). Mice were euthanized with sodium pentobarbital i.p., urine was collected, and blood drawn from the right ventricle of the heart. The lungs were perfused with saline through the right ventricle until they blanched. Next, the systemic circulation was perfused with saline through the left ventricle until the liver and kidneys had blanched noticeably. Out of each group of 4-5 mice, 1-2 had their organs removed at this point; the remaining three of each group had their lungs inflation-fixed with 0.8 mL of 10% neutral buffered formalin (NBF), whereas the systemic circulation was subsequently perfused with 10% NBF to fix the other organs, after which these organs were removed as well. The contents of the stomach, small intestine, and colon were thoroughly flushed out with saline before weighing and gamma counting.

The following tissues, fluids, and organs were assayed: blood, urine, stomach, small intestine, colon, bladder, kidneys, pancreas, spleen, liver, skeletal muscle, bone (femur), thymus, heart, lungs, and brain. These were dispensed into previously-tared gamma counting vials and weighed. Samples were kept on ice until ready for counting. Radiolabeled protein biodistribution at various time points and routes of administration was reported as the ratio between organ radioactivity and organ weight (microcuries per gram, $\mu\text{Ci/g}$). For lung, the clearance kinetics of the i.t.-administered radioactive tracer were determined by mono-exponential fit of the organ

radioactivity/weight ratios. Following gamma counting, unfixed samples were transferred to tubes and flash frozen in liquid nitrogen, then transferred to -80°C for long term storage; fixed organs, excepting bone, were placed in cassettes and agitated in a large volume of 10% NBF overnight; bone was simultaneously decalcified and fixed in Cal-Rite (Richard Allen scientific) overnight. Following fixation, cassettes were transferred into 85% ethanol and thus stored until ready for paraffin embedding.

3.1.6 Exogenous sRAGE-induced eosinophil chemotaxis study

To test the potential eosinophil-recruiting effect of sRAGE, eight-week-old male wild type mice were treated i.t. with 50 µg of MSA or an equal mass of sRAGE dissolved in 70 µL of PBS. Mice were sacrificed at 24, 48, and 96 hours after treatment. To test for the possibility that an induction dose is necessary to prime the lung compartment for enhanced responsiveness to subsequent doses, mice were also treated on days 0 and 1 with 50 µg of MSA or sRAGE (on each treatment day) and sacrificed on day 3.

3.1.7 Models of allergic airway disease/asthma

Allergic airway disease/asthma was induced in eight-week-old male wild type or RAGE KO mice using one of three protocols (Figure 2). In the first protocol (Figure 2A), mice were treated intranasally (i.n.) four times per week for seven weeks with 40 µg of house dust mite (HDM) extract in 25 µL of saline. Control mice were treated with saline vehicle alone. Mice were sacrificed 48 hours after the last treatment.

The second protocol (Figure 2B) used ovalbumin (OVA) as the sensitization/challenge antigen. Sensitization was effected by i.p. treatment with 50 µg of ovalbumin with 2 mg of aluminum hydroxide gel (alum, Brenntag) in 0.5 mL of saline on days 0 and 7. Control mice were given alum with saline alone during the sensitization phase. Intranasal challenge commenced at day 14 with 10 µg of ovalbumin in 25 µL of saline, continuing on alternate days for a total of three treatments per week for three weeks. Control mice were given saline alone during the challenge phase. Mice were sacrificed 24 hours after the last treatment.

In the third protocol (Figure 2C), wild type mice were treated i.n. five times per week for three weeks with one of the following six treatments (in each case prepared in 25 µL of phosphate buffered saline (PBS)): saline control, 25 µg MSA alone, 25 µg mouse sRAGE alone, 40 µg HDM extract alone, 40 µg HDM extract with 25 µg MSA, or 40 µg HDM extract with 25 µg sRAGE. Mice were sacrificed 24 hours after the final treatment. Pulmonary function testing was performed in these mice.

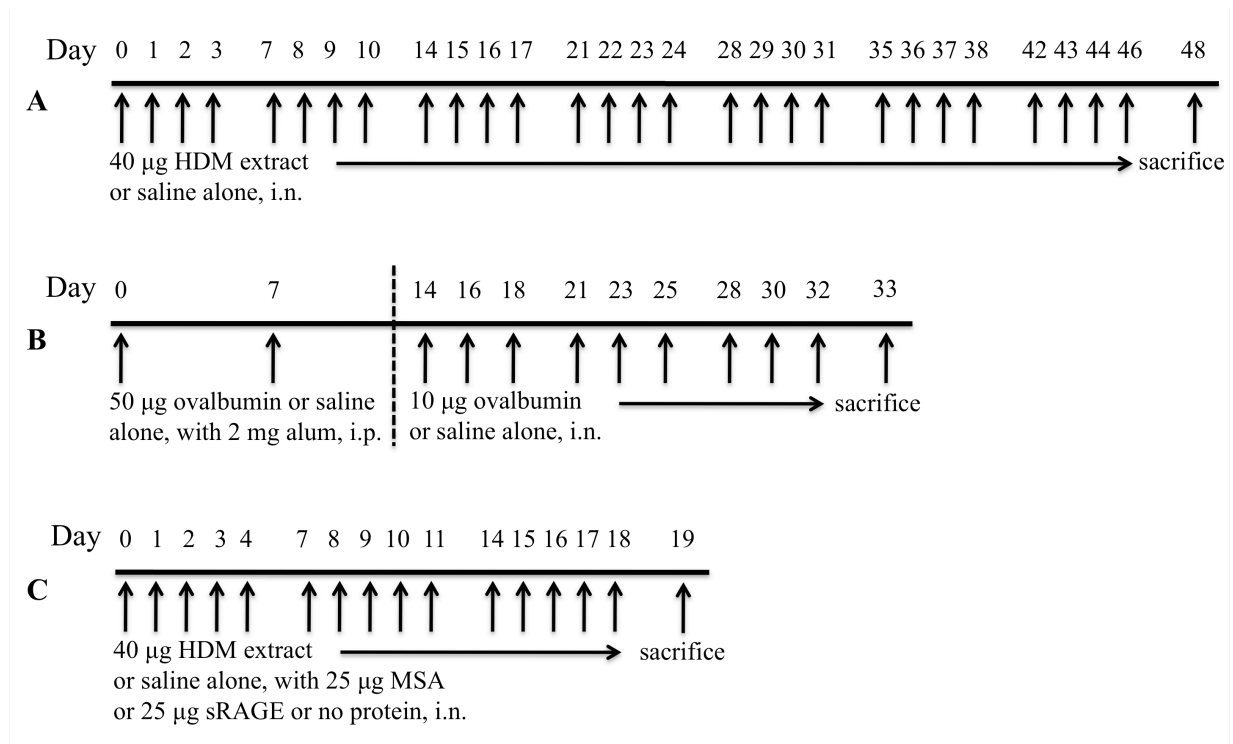


Figure 2. Experimental asthma mouse model sensitization/challenge protocols.

(A) A seven-week house dust mite (HDM) extract sensitization/challenge protocol. (B) A five-week ovalbumin (OVA) sensitization and challenge protocol. (C) A three-week HDM extract sensitization/challenge protocol involving the use of co-administered purified proteins.

3.1.8 Generation of bone marrow chimeras

Eight-week-old male wild type or congenic RAGE KO recipient mice were administered 0.032 mg/mL of enrofloxacin (Bayer) in drinking water two days prior to bone marrow transplant and kept on antibiotics until seven days post-transplant. Donor wild type and RAGE KO mice were euthanized and tibias and femurs harvested into cold Hank's balanced salts solution (HBSS) containing penicillin and streptomycin. The marrow was flushed out of the bones with a needle and syringe, and the marrow cells were disrupted into a suspension, then strained to remove bone fragments and undisrupted marrow, and washed several times with antibiotics- and serum-free cold HBSS, then counted. Recipient mice were given 10 Gy (1000 R) of whole-body ionizing

radiation in one dose and received $15\text{--}20 \times 10^6$ bone marrow cells in $\sim 200 \mu\text{L}$ HBSS by tail vein injection within 8 hours of the irradiation.

Four weeks following the bone marrow transplantation, mice were given liposomal clodronate i.t. to ablate any radioresistant pulmonary macrophages in the recipients.

3.1.9 Ablation of pulmonary macrophages with liposomal clodronate

Bisphosphonate dichloromethylenediphosphonic acid disodium (clodronate) encapsulated in liposomes was administered to selectively ablate pulmonary macrophages that had possibly survived irradiation.²⁸⁸ Eight milligrams (8 mg) of cholesterol (Sigma) and 86 mg of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids) were dissolved in chloroform in a glass vial. The solvent was allowed to evaporate by bubbling nitrogen into the solution over several hours to generate a dry hydrophobic cake on the inside surface of the vial. One gram (1 g) of clodronate (Sigma) was prepared in 5 mL of sterile PBS and added to the lipophilic contents of the vial. Liposomes were generated by vortexing the vial contents for 30 minutes and then sonicating for 5 minutes. Centrifugation for 1 hour at $125,000 \times g$ effected phase separation, with liposomes floating at the surface. The clodronate solution was aspirated and the liposomes were washed by resuspension and mixing into 5 mL of sterile PBS. Centrifugation was repeated as previously, and the PBS supernatant was aspirated. The liposomes were then reconstituted in 4 mL of sterile PBS and administered i.t. at a volume of $75 \mu\text{L}$ to each of the bone marrow chimeric mice.

Four weeks following the treatment with liposomal clodronate, the mice were subject to the first of the three protocols outlined in the subsection above (Figure 2A). Pulmonary function testing was performed in these mice.

3.1.10 IL-33 release study

To test the capacity of HDM extract to induce release of IL-33 from intracellular stores, eight-week-old male wild type mice were treated i.t. with 40, 80, or 120 μ g of HDM extract dissolved in 70 μ L of PBS, with PBS vehicle as the control treatment. Mice were sacrificed at one of the following time points after treatment: 30 minutes, 1, 2, 3, or 4 hours.

3.1.11 Pulmonary function testing

Pulmonary function was assayed using the forced oscillation technique on a flexiVent apparatus as described elsewhere.²⁸⁹ Briefly, mice were anesthetized with sodium pentobarbital (~100 mg/kg), tracheostomized, cannulated and coupled to a flexiVent ventilator apparatus (SCIREQ). Mice were ventilated with a 0.2 mL tidal volume and PEEP of ~3 cm H₂O. Pressure and volume were measured to generate pressure-volume curves, which were then fit by multiple linear regression to a mathematical model of the lung. Methacholine (Sigma) was delivered via a nebulizer; following each dose, the response was measured by applying 2-second perturbations at 10-second intervals for a total of 3 minutes. Dose-response curves were then determined for each of three parameters measuring lung function: Newtonian resistance (R_n), tissue damping (G), and elastance (H).

3.1.12 Processing of animals in studies not involving radioactive substances

In studies in which pulmonary function testing was not performed, the mice were first euthanized with sodium pentobarbital (~250 mg/kg), tracheostomized, and cannulated (in studies involving

pulmonary function testing, mice were already anesthetized and cannulated in order to test pulmonary mechanics).

Mice were exsanguinated and sera prepared using serum separator tubes (Becton Dickinson); following coagulation, serum tubes were centrifuged at 15,000 x g for 5 minutes, and the serum was collected and transferred to fresh tubes.

Eight hundred microliters (0.8 mL) of saline was instilled in the lungs via the trachea and withdrawn. Bronchoalveolar lavage fluid (BALF) was diluted twofold for cell counts and cytopsin slides were prepared for differential cell counting using a cytocentrifuge (Shandon). Slides were air dried, stained with Diff-Quik (Siemens), air dried again, and coverslipped with Permount (Fisher). Cells per milliliter of BALF were determined manually by counting all hematopoietic cells in each of eight high power fields, determining the average number of cells per field, multiplying by a factor relating the area of a high power field to the total area of the cytopsin disk (x144), and finally multiplying by an appropriate dilution factor taking into account the dilution of BALF (x2) and the quantity of diluted BALF loaded for each cytopsin slide (100 μ L, hence x10 for cells/mL). The relative percentage of each of monocytes, eosinophils, neutrophils, and lymphocytes was determined by counting five high power fields. Remaining BALF was centrifuged at 10,000 x g for 10 minutes to sediment cells suspended in BALF, and the BALF supernatant was transferred to fresh tubes.

The right lung was tied off with suture, removed, divided in several pieces into separate tubes (for various downstream analyses, e.g. immunoblotting, frozen tissue embedding and sectioning or quantitative reverse transcription polymerase chain reaction (qRT-PCR)), and flash frozen in liquid nitrogen. The left lung was inflation fixed in the chest cavity with 0.6 mL of 10% NBF for ~10 minutes, excised and transferred to tissue cassettes for 4 hrs of fixation in NBF,

then transferred to 70% ethanol until ready for paraffin embedding by the University of Pittsburgh's Research Histology Services.

BALF supernatants, BALF cell pellets, sera, and lungs that had previously been flash frozen in liquid nitrogen were stored at -80°C for downstream analyses.

3.2 BIOCHEMICAL ANALYSES

3.2.1 Tissue homogenization for protein analyses

Lung homogenates were used in a variety of downstream applications, including SDS-PAGE followed by gel autoradiography, SDS-PAGE followed by membrane transfer and immunoblotting, sRAGE affinity chromatography, and immunoglobulin or cytokine enzyme-linked immunosorbent assay (ELISA).

For lung homogenates in which membrane proteins as well as soluble proteins were desired, frozen lungs were homogenized in cold CHAPS buffer (150 mM NaCl, 50 mM Tris-HCl, 10 mM CHAPS, pH 7.4) with protease inhibitors (all from Sigma: 100 μ M 3,4-dichloroisocoumarin (DCI), 10 μ M *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), 2 mM o-phenanthroline monohydrate), followed by brief sonication, and then protein extraction over 2-4 hours at 4°C. For lung homogenates in which only soluble proteins were desired, CHAPS detergent was withheld from the buffer and sonication was omitted. Following extraction, insoluble substances were sedimented by centrifugation at 20,000 x g for 20 minutes at 4°C. The supernatants were transferred to fresh tubes and stored at -80°C until further use.

3.2.2 Determination of protein content

Total protein content in lung homogenates was determined using the Bradford method using Bradford reagent (Thermo Fisher), serially-diluted bovine serum albumin (BSA) standard protein (Pierce) to generate a standard curve, and a SpectraMax plate reader (Molecular Devices). Briefly, fixed volumes of protein samples were mixed with fixed volumes of Bradford reagent in 96-well plates, incubated, and absorbances read at 595 nm on the plate reader. Total protein concentrations of samples were determined by comparison of measured absorbances to the standard curve relating known concentrations of BSA to absorbances at the same wavelength.

3.2.3 sRAGE affinity chromatography

Cyanogen bromide-activated Sepharose 4B resin (Sigma) was reconstituted in cold 1 mM HCl, then washed five times with a 10X volume of distilled water. The resin was then washed into coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.5) and incubated with concentrated purified mouse sRAGE in coupling buffer overnight at 4°C. The resin was then sedimented, washed in coupling buffer, and incubated in 0.2 M glycine, pH 8.0, overnight at 4°C to quench unreacted cyanogen bromide sites. Finally, the resin was washed four times with alternating 20X volumes of coupling buffer and acetate buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 4.0), loaded into a glass column fitted with a glass frit, and stored in 1 M NaCl at 4°C until further use. The resin-coupled sRAGE was shown to retain specific HMGB1 binding activity.

To probe HDM extract for RAGE ligands, 15 mg of HDM extract (corresponding to ~5 mg of HDM extract protein) was diluted in loading buffer (50 mM HEPES, 50 mM NaCl, pH 7.4) and passed over the sRAGE-coupled resin. To probe lung homogenates derived from mouse

models of allergic airway disease/asthma for RAGE ligands, ~2-3 mg of total protein from pooled lung homogenate (lacking CHAPS detergent) from each strain/treatment group was diluted into loading buffer and passed over the sRAGE-coupled resin. To elute loosely-bound molecules, a more physiologic washing buffer (50 mM HEPES, 150 mM NaCl, pH 7.4) was used to wash the resin. Finally, to elute the sRAGE-bound molecules, high salt elution buffer (50 mM HEPES, 1 M NaCl, pH 7.4) was applied to the resin. The eluate was concentrated using 3000 kDa molecular weight cutoff centrifugal filter units (Amicon).

3.2.4 Identification of RAGE ligands by mass spectrometry

Samples were denatured with 6 M urea and reduced with 5 mM dithiothreitol (DTT) in a 20 mM Tris-HCl, pH 8 buffer. Alkylation was performed with 15 mM iodoacetamide and following dilution in 20 mM ammonium bicarbonate buffer the samples were digested with trypsin (w/w: 1:25) at 37°C for 16 hours. Tryptic peptides were purified per manufacturer instructions on StageTips (Thermo Scientific) and then lyophilized. Nano-electrospray ionization tandem mass spectrometry (nanoESI-MS/MS) was performed on an EASY-nLC II system (Thermo Scientific) coupled to a TripleTOF 5600 mass spectrometer (AB Sciex), equipped with a NanoSpray III source (AB Sciex) under Analyst TF 1.5.1 control. The tryptic digests were suspended in 0.1% formic acid and injected, trapped and desalted on a ReproSil-Pur C18-AQ column (5 μ m, 2 cm x 100 μ m I.D; Thermo Scientific). Peptides were eluted from the trap column and separated on a ReproSil-Pur C18-AQ capillary column (3 μ m, 10 cm x 75 μ m I.D; Thermo Scientific) connected in-line to the mass spectrometer at 250 nl/min, using a 50-minute gradient from 5% to 35% phase B (0.1% formic acid and 90% acetonitrile). Data processing was performed using Peakview 1.0 to generate MGF files. Files were searched using the Mascot search engine (Matrix

Science) against an in-house database with MS/MS tolerance set to 0.1 Da and peptide tolerance set to 10 ppm.

To lend the data a semi-quantitative footing, exponentially-modified protein abundance index (emPAI) was used for comparison between samples. Protein abundance index (PAI) normalizes the number of observed peptides to the number of observable peptides for each protein species.²⁹⁰ emPAI, which is defined as $10^{\text{PAI}} - 1$, in turn, is proportional to the abundance of a protein species in a mixture.²⁹¹ Because of intersample variations in the initial pooled raw homogenate total protein content loaded over the sRAGE-conjugated resin, further analysis of emPAI values was performed, such that the sum of emPAI values for each pooled sample was determined (this estimates total protein loaded) and individual emPAI values for each species were normalized to this sum. This is briefly summarized in the formula below (where i represents an individual protein species, N represents the total number of peptides (actually observed or theoretically observable), and n represents the total number of protein species detected in the mixture).

$$\text{Normalized emPAI}_i = \frac{10^{\frac{N_i(\text{peptides observed})}{N_i(\text{peptides observable})} - 1}}{\sum_i^n \left(10^{\frac{N_i(\text{peptides observed})}{N_i(\text{peptides observable})} - 1} \right)}$$

3.2.5 SDS-PAGE, gel staining, and gel autoradiography

For SDS-PAGE analysis of purified unlabeled MSA or sRAGE, samples were adjusted to an equal concentration and volume, whereas radiolabeled MSA or sRAGE were assessed by gamma counting and adjusted to an equal activity and volume prior to gel separation. Frozen lungs from

mice that had been treated i.t. with radiolabeled MSA or sRAGE were homogenized in 1.5 mL cold buffer lacking CHAPS detergent and containing protease inhibitors, as described previously; sixty microliters (60 μ L) of each sample was used for SDS-PAGE separation.

For SDS-PAGE separation of HDM extract components, 100 μ g of HDM extract was used for each lane. For animal studies that did not employ radioactive substances, uniform quantities (5-35 μ g) of total lung homogenate protein, 65 μ L of undiluted BALF, and/or 65 μ L of tenfold diluted (in PBS) serum samples were applied to each lane.

For SDS-PAGE analysis of RAGE-binding molecules, whether derived from HDM extract or from lung homogenates, the complete eluates, which had first been concentrated by centrifugal filtration, were used.

Samples were denatured and reduced with SDS and 50 mM DTT, then boiled for 5 minutes and shock-cooled on ice. DTT was omitted in some cases when HDM extract was prepared for SDS-PAGE under non-reducing conditions. Samples were separated on 5-15% 2-amino-2-methyl-1,3-propanediol-buffered polyacrylamide gradient gels as described previously.²⁹² Gels with known or anticipated microgram quantities of protein were stained with Coomassie Brilliant Blue and destained in a methanol/acetic acid-containing solution; gels were imaged with simple white light transillumination. For gel autoradiography, gels were incubated in a 3% glycerol-containing destain solution for 1 hour, then dried overnight on cellulose membranes. Dried gels were incubated at -80°C in a metal cassette with photographic film (Amersham Biosciences), and exposed film was thereafter developed and scanned. Gels with known or anticipated nanogram quantities of protein were stained with SYPRO Ruby protein gel stain (Invitrogen) and destained per manufacturer instructions; gels were imaged with UV transillumination using a 595 nm emission filter. Gels were visualized on a Kodak Gel Logic

2200 Imaging System and recorded using Kodak Molecular Imaging software (both from Kodak).

3.2.6 Immunoblotting

Gels were equilibrated and transferred to PVDF membranes in immunoblotting transfer buffer containing 6-aminohexanoic acid and methanol at 500 mAmps for 1 hr with cooling. Following transfer, membranes were incubated in Ponceau S staining solution, destained in methanol/acetic acid-containing solution, and recorded by scanning. Membranes were reactivated with methanol, and acetic acid was washed out with PBS containing Tween-20 (PBST) three times, at 10 minutes per wash. Following overnight blocking in 5% milk in PBST at 4°C, membranes were washed three times with PBST at 5 minutes per wash, and then probed with one of the following (1 hour for each primary anti-mouse antibody incubation at room temperature, unless noted otherwise, and three washes in PBST at 5 minutes per wash after each antibody incubation): 1:5000 rabbit anti-RAGE polyclonal antibody (GenScript 95131-9B) in PBST followed by 1:5000 secondary horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch) in 1% milk in PBST; 1:5000 mouse anti- β -actin monoclonal antibody (Sigma A5316) in PBST followed by 1:10000 secondary HRP-conjugated rabbit anti-mouse IgG antibody (Jackson ImmunoResearch) in PBST; 1:20 diluted (into PBST) pooled serum, obtained from wild type or RAGE KO mice sensitized/challenged with HDM extract, overnight at 4°C, followed by 1:5000 secondary HRP-conjugated rabbit anti-mouse IgG antibody in 1% milk in PBST; 1:500 goat anti-mouse IL-33 polyclonal antibody (R&D Systems AF3626) in PBST followed by 1:5000 secondary HRP-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch) in 1% milk in PBST.

Membranes were developed using enhanced chemiluminescent detection (Thermo-Fisher). Developed membranes in all cases were visualized on a Kodak Gel Logic 2200 Imaging System and recorded using Kodak Molecular Imaging software. Loading was controlled using Ponceau S staining for BALF and serum, and for lung homogenate blots by membrane stripping, overnight blocking, and re-probing for β -actin as described above. Membrane stripping was performed by incubating the membrane twice in succession for 30 minutes in stripping buffer (25 mM glycine, 1% SDS, pH 2), followed by three washes at 10 minutes per wash in PBST prior to blocking overnight.

Densitometric analysis was performed using the same software used to record the images. For each sample, net signal intensity was determined for both RAGE (or IL-33) bands and the respective β -actin bands. The RAGE: β -actin (or IL-33: β -actin) signal intensity ratios for each sample were determined (in the case of RAGE, for each individual isoform), with the mean ratio for saline-treated controls arbitrarily set to 1.0 and used to normalize all other ratios.

3.2.7 RNA isolation and qRT-PCR

RNA was prepared from whole lung using an RNeasy Mini Kit (Qiagen), per manufacturer instructions. Nuclei acid concentration was determined using the following formula: absorbance at 260 nm x dilution factor x 40; the degree of protein contamination was evaluated using the ratio of absorbance at 260 nm to absorbance at 280 nm. Reverse transcription (RT) was performed with 1 μ g of each sample's RNA, using the following mastermix: 2.5 U MuLV reverse transcriptase, 5 mM $MgCl_2$, 1X PCR Buffer II, and 1 mM dNTPs (all from Applied Biosystems) in a thermocycler (Techne). The following program was used: 42°C for 40 minutes, 99°C for 5 minutes, and 5°C for 5 minutes. Quantitative PCR (qRT-PCR) was performed using

2X universal TaqMan PCR buffer and TaqMan primer/probe assay reagent (both from Applied Biosystems) with primers for RAGE (Mm00545815_m1), IL-5 (Mm00439646_m1), IL-13 (Mm00505403_m1), TSLP (Mm00498739_m1), IL-33 (Mm00505403_m1), and GAPDH control (Mm99999915_g1). The following sequence was performed on an ABI Prism 7300 machine (Applied Biosystems): 50°C (2 minutes), 95°C (10 minutes), and then 40 cycles of 95°C (15 seconds) followed by 60°C (1 minute). The fold change in RAGE, IL-5, IL-13, TSLP, or IL-33 mRNA expression, as compared to GAPDH mRNA housekeeping control, was determined using the $\Delta\Delta C_t$ method, with wild type saline-treated control mice set at a fold change of 1.0.

3.2.8 ELISA

ELISA was used to assess either the immunoglobulin response or cytokine expression in mouse models of allergic airway disease/asthma. HDM-specific IgG₁ ELISA was performed by coating high-binding polystyrene 96 well plates with 2 µg/mL HDM extract in coating buffer (100 mM NaHCO₃, 30 mM Na₂CO₃, pH 9.5) overnight at 4°C. Between each step, washes were performed with PBST. After blocking with 1% BSA for 2 hours, 1:100 diluted serum or neat lung homogenate (containing CHAPS detergent) were applied and incubated at 4°C overnight. Detection was performed by probing with 1:1000 secondary HRP-conjugated goat anti-mouse IgG₁ (Jackson ImmunoResearch) for 1 hour at room temperature, and then incubating with o-phenylenediamine (OPD) substrate (Sigma) for 30 minutes. Plates were read at 450 nm and data are recorded as absolute absorbances. Total IgE ELISA was performed using 1:20-diluted serum and an OptEIA IgE ELISA kit (BD Biosciences) per manufacturer instructions. To detect HDM-binding IgE, first HDM extract was biotinylated using biotin-X,SSE (Invitrogen); then, free

biotin was removed using a Sephadex G-25 quick spin column (Roche) and biotinylated HDM extract was eluted and stored at -80°C until further use. HDM-specific IgE ELISA was performed by first using the OptEIA IgE ELISA kit to capture total IgE in 1:5-diluted serum, overnight at 4°C. Then, biotinylated HDM extract was applied for 2 hours at room temperature, followed by washing, application of HRP-conjugated streptavidin from the IgE ELISA kit for 1 hour, and then detection by incubating with OPD substrate for 30 minutes. For all immunoglobulin ELISAs the plates were read at 450 nm and data were recorded as absolute absorbances or, in the case of total IgE, the concentration of immunoglobulin was determined by comparing sample absorbances to a standard curve relating known concentrations of manufacturer-supplied IgE standard to absorbances at the same wavelength.

ELISAs for eotaxin and eotaxin-2 (both from Abcam); IL-4 and IL-5 (both from BD Biosciences); IL-17 (R&D Systems); IL-13, IL-25, TSLP, and IL-33 (all from eBioscience) were performed per manufacturer instructions, using neat or diluted BALF in all cases except for IL-17 and TSLP, which utilized neat lung homogenate. Samples were incubated overnight at 4°C, with agitation. Blocking and detection antibody incubation steps were generally performed for 1-2 hours at room temperature, whereas colorimetric detection incubation steps (whether with OPD or 3,3',5,5'-tetramethylbenzidine (TMB)) were performed for 15-30 minutes in the dark. The plates were read at 450 nm and absolute concentrations of cytokines were determined by comparing sample absorbances to a standard curve relating known concentrations of manufacturer-supplied cytokine standard to absorbances at the same wavelength.

3.3 HISTOLOGIC ANALYSES

3.3.1 Tissue embedding, sectioning, and preparation for downstream applications

Tissue embedding in paraffin was performed in the lab for specimens containing radioactive substances. For specimens not containing radioactive substances, formalin-fixed lungs stored in ethanol were submitted to the University of Pittsburgh's Research Histology Services for dehydration through ethanol, pre-clearing in xylenes, and embedding in paraffin. For immunohistofluorescence staining, blocks were sectioned on a microtome to a 5- μ m thickness, adsorbed to Superfrost Plus glass slides (Fisher Scientific), dried, incubated at 60°C to improve adherence to the slide, deparaffinized in three 10-minute washes in xylenes, and rehydrated through a series of 100%, 95%, 90%, 85%, and 70% ethanol to water.

For frozen tissue embedding, flash frozen lung was immersed in optimal cutting temperature (OCT) media (Tissue-Tek) and frozen with liquid nitrogen into molds suitable for mounting blocks into a cryomicrotome. Cryosections were obtained at an 8- μ m thickness and adsorbed to Superfrost Plus glass slides and stored at -80°C with desiccant until further use. To prepare sections for immunofluorescence staining, slides were allowed to equilibrate to room temperature for 20 minutes, then fixed for 5 minutes in dry 75% acetone/25% ethanol that had previously been cooled to -20°C, and then left to dry again at room temperature.

3.3.2 Tissue section autoradiography

All steps were undertaken under very low (red) light conditions until the fixation step. Autoradiography emulsion NBT (Kodak) was melted at 42°C in a warm water bath. Five

micrometer-thickness lung sections previously mounted on slides, deparaffinized, and rehydrated, were dipped in molten emulsion, briefly air dried, and stored in light-insulated slide boxes with desiccant. The boxes were sealed to prevent entry of water, wrapped in foil, and stored at 4°C in the dark for 3-5 weeks. Following incubation, the slides were developed in D-19 developer and fixed in fixer (both from Kodak), then washed in water, and counterstained, as described in the subsequent subsection.

3.3.3 Histochemical staining

For hematoxylin and eosin (H&E) counterstaining of NTB emulsion-dipped radioactive lung sections (silver grains having been already developed and fixed), specimens were dipped into Gill's hematoxylin (Vector Labs) for up to 15 minutes to generate an adequate counterstain, then dehydrated through ethanol, briefly dipped in eosin Y for several seconds, pre-cleared in xylenes, and coverslipped with Permount hydrophobic mounting media (Fisher Scientific).

For histochemical staining of non-radioactive specimens, paraffin blocks were submitted to the University of Pittsburgh's Research Histology Services for sectioning, slide adsorption, deparaffinization, and rehydration. Sections were stained with H&E stain and in some cases periodic acid-Schiff (PAS) stain. In all cases, sections were examined by light microscopy on an Olympus BH-2 microscope and photomicrographs were obtained using an Olympus DP12 camera. All images were processed using ImageJ software.²⁹³ Images were light background-subtracted; in all cases a rolling ball radius of 50.0 pixels was used.

3.3.4 Immunofluorescence microscopy

Lung sections were mounted, dried, melted, deparaffinized, and hydrated as described previously. Whenever possible, RAGE KO sections were used as controls for RAGE staining, and isotype matched antibodies against irrelevant antigens and pre-immune sera were used as controls for staining of other markers. For immunofluorescence detection of RAGE (in frozen sections) and the T lymphocyte marker CD4, no antigen retrieval was necessary. For immunofluorescence detection of RAGE in paraffin sections, the basement membrane collagen IV, the eosinophil marker major basic protein (MBP), and the macrophage marker F4/80, antigen retrieval was performed in 0.2 N HCl with 1 mg/mL pepsin (Fisher) at 37°C for 10 minutes. For immunofluorescence detection of VCAM-1, IL-33, the bronchial epithelial cell marker uteroglobin/Clara cell secretory protein (CCSP), the endothelial cell marker CD31/PECAM-1, the type I alveolar epithelial cell marker aquaporin 5 (AQP5), the type II alveolar epithelial cell marker prosurfactant protein C (proSP-C), or the smooth muscle cell marker α -smooth muscle actin (α -SMA), antigen retrieval was performed in boiling citrate buffer (Dako) at pH 6 for 30 minutes. Following antigen retrieval (or, for frozen sections post-fixation drying), sections were washed twice in PBS at 5 minutes per wash, and a hydrophobic barrier pen was used to encircle each of the sections, which were then blocked with 2% BSA in PBST for 1 hour at room temperature.

Following blocking, sections were washed three times with PBST at 5 minutes per wash, and then probed with one or two of the following primary antibodies (incubation conditions of 1 hour at room temperature or overnight at 4°C for each primary anti-mouse antibody (diluted in 0.5% BSA in PBST)): 1:500 goat anti-RAGE antiserum (GenScript), 1:500 rabbit anti-RAGE polyclonal antibody (GenScript 35131-9B), 1:500 rabbit anti-collagen IV polyclonal antibody

(Pierce PA1-26148) 1:500 rat anti-MBP monoclonal antibody (kindly provided by the Dr. J. Lee, Mayo Clinic), 1:50 rat anti-CD4 monoclonal antibody (clone RM4-5, eBioscience 14-0042), 1:100 rat anti-F4/80 monoclonal antibody (clone A3-1, Abcam ab6640), 1:500 goat anti-VCAM-1 polyclonal antibody (R&D Systems AF643), 1:100 anti-IL-33 polyclonal antibody (R&D Systems AF3626), 1:2000 rabbit anti-CCSP antiserum (Millipore 07-623), 1:50 rabbit anti-CD31 polyclonal antibody (Abcam ab28364), 1:100 rabbit anti-AQP5 polyclonal antibody (Millipore AB15858), 1:4000 rabbit anti-proSP-C polyclonal antibody (Millipore AB3786), or rabbit anti- α -SMA polyclonal antibody (Abcam ab5694).

Following single or dual primary antibody incubation, sections were washed three times with PBST at 5 minutes per wash, and then probed with appropriate single secondary antibody or pairs of secondary antibody (diluted in 0.5% BSA in PBST) for 1 hr at room temperature in the dark: DyLight 488 (henceforth “DL488”)- or AlexaFluor 488 (henceforth “AF488”)- or Cyanine 3 (Cy3)-conjugated anti-rabbit or anti-goat or anti-rat polyclonal antibody (all from Jackson ImmunoResearch). In all cases secondary antibodies were raised in donkey and, particularly for those raised against rat IgG, care was taken to purchase antibodies that exhibit minimal cross-reactivity to mouse IgG. Following secondary antibody incubation, sections were washed three times with PBST and then twice with PBS at 5 minutes per wash. Nuclei were stained using a brief application of Hoechst stain (10 mg/mL), and free Hoechst stain was washed out with three washes of PBS at 1 minute per wash. Sections were coverslipped with gelvatol (recipe from the Center for Biologic Imaging at the University of Pittsburgh: 21 g of polyvinyl alcohol, 42 mL of glycerol, 52 mL of water, 106 mL of 0.2 M Tris-HCl at pH 8.5, and a few crystals of sodium azide; reagents dissolved by heat and agitation; viscosity adjusted by the addition of glycerol or polyvinyl alcohol; clarified by centrifugation at 5,000 x g for 15 minutes; stored at 4°C),

examined by an Olympus IX71 inverted microscope, and representative photomicrographs obtained. All images were processed using ImageJ software, with images background-subtracted as individual colors, followed by color channels being merged; in all cases a rolling ball radius of 50.0 pixels was used.

3.3.5 Histologic scoring

H&E-stained lung sections from wild type mice subjected to the third of the enumerated sensitization/challenge protocols modeling allergic airway disease/asthma (Figure 2C), were examined by a board certified pathologist (Tim D. Oury, MD, PhD), who had been blinded to the identity of the samples. The total number of bronchovascular bundles in each lung section was determined and inflammation was scored. Inflammation in each bronchovascular bundle was graded as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The results are expressed as the percentage of all bronchovascular bundles that involve any inflammatory infiltrates (score of 1 or above), and additionally as an average severity score obtainable by dividing the pan-section sum of the bronchovascular bundle inflammation scores by the total number of bronchovascular bundles in that section.

Lung sections from wild type or RAGE KO mice subjected to the first of the allergic airway disease/asthma model sensitization/challenge protocols summarized previously (Figure 2A), stained for the presence of IL-33 and visualized by immunofluorescence microscopy, as described previously, were scored for the density of IL-33⁺ nuclei/cells. Staining conditions, and the parameters of imaging and image processing were identical for all samples. Briefly, five randomly-selected primarily-parenchymal areas of lung section from each individual mouse lung specimen were inspected at 10X magnification and recorded separately for both Cy3 signal

(corresponding to IL-33⁺ nuclei/cells) and Hoechst signal (corresponding to all nuclei). The photomicrographs were background-subtracted on ImageJ software as previously described, color-inverted, and converted to 8-bit grayscale files. These images, in turn, were analyzed using the Image-Based Tool for Counting Nuclei (ITCN) plug-in (Center for Bio-Image Informatics, University of California, Santa Barbara, CA) in ImageJ. For each corresponding pair of IL-33 signal and Hoechst signal images, the ratio between the number of IL-33⁺ nuclei/cells and the total number of nuclei/cells was determined. From these ratios, the mean for each sample was determined, and from these latter values, the mean for each strain/treatment group was determined. The mean ratio for wild type saline control-treated mice was arbitrarily set at 1.0 and was used to normalize the other strain/treatment group ratios.

3.4 REGRESSION AND STATISTICAL ANALYSES

Quantitative data were analyzed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). Linear regression for standard curves and exponential decay fitting for radioactive tracer clearance were performed using this program, as were statistical analyses. Comparisons involving one variable were conducted with Student's t-test, whereas comparisons involving two variables, such as those pertaining to experiments varying both strain and treatment, were performed with 2-way analysis of variance (2-way ANOVA) followed by post-hoc t-test. All values are reported as mean \pm standard error of the mean (S.E.M.). A p-value of $p < 0.05$ was considered statistically significant. Sample sizes are indicated in figure captions.

4.0 RESULTS

4.1 INTRATRACHEAL INSTILLATION, BUT NOT INTRAVENOUS OR INTRAPERITONEAL INJECTION, DELIVERS SOLUBLE RAGE TO THE LUNG

To determine the most efficacious means of delivery of sRAGE to the lung, biodistribution and clearance studies were performed with radiolabeled sRAGE or MSA (as a protein control) given via three common routes of administration: i.v. injection, i.p. injection, or i.t. instillation. To confirm that the proteins being administered were relatively pure, SDS-PAGE and Coomassie Brilliant Blue gel staining were performed and indeed failed to demonstrate the presence of proteins other than MSA (~69 kDa) and sRAGE (~37-42 kDa), which migrated as expected (Figure 3A). Following labeling with ^{125}I , SDS-PAGE and gel autoradiography was performed to confirm incorporation of the label into sRAGE or MSA and removal of free radiolabel (Figure 3B). Some slight radiolabeled impurities are evident, particularly in the sRAGE preparation, but are unlikely to account for more than a few percents of the total protein given.

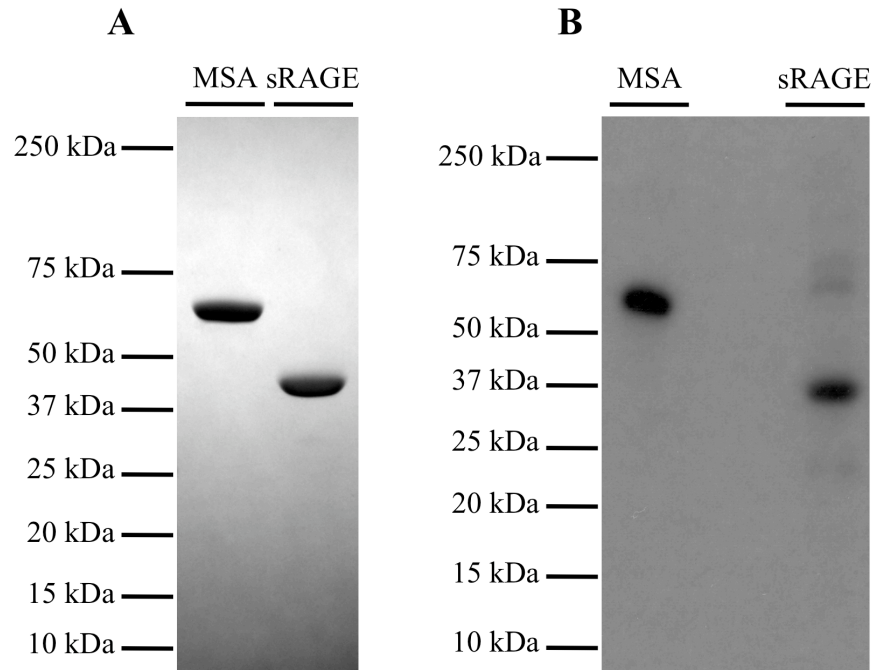


Figure 3. sRAGE and MSA used in biodistribution and clearance studies are relatively pure.

(A) SDS-PAGE separation and Coomassie Brilliant Blue staining of ~2.5 µg of MSA or sRAGE preparations used for radioiodination, and in later experiments, testing the potential eosinophil-recruiting effect of sRAGE. (B) SDS-PAGE separation and gel autoradiography of ¹²⁵I-labeled MSA and sRAGE.

Small quantities of radiotracer were used so as to minimize any potential effect of these bioactive proteins on the baseline physiology of the animals. Intravenous and intraperitoneal injection failed to deliver an appreciable quantity of sRAGE or MSA protein control to the lung (Figures 4 and 5, respectively). Distribution to the lung failed to occur within the 12-hour window of investigation, by which time extensive renal clearance had already occurred. In contrast, intratracheal instillation was very efficacious at delivering sRAGE and MSA to the lung, and was in fact the only feasible means of doing so (Figure 6).

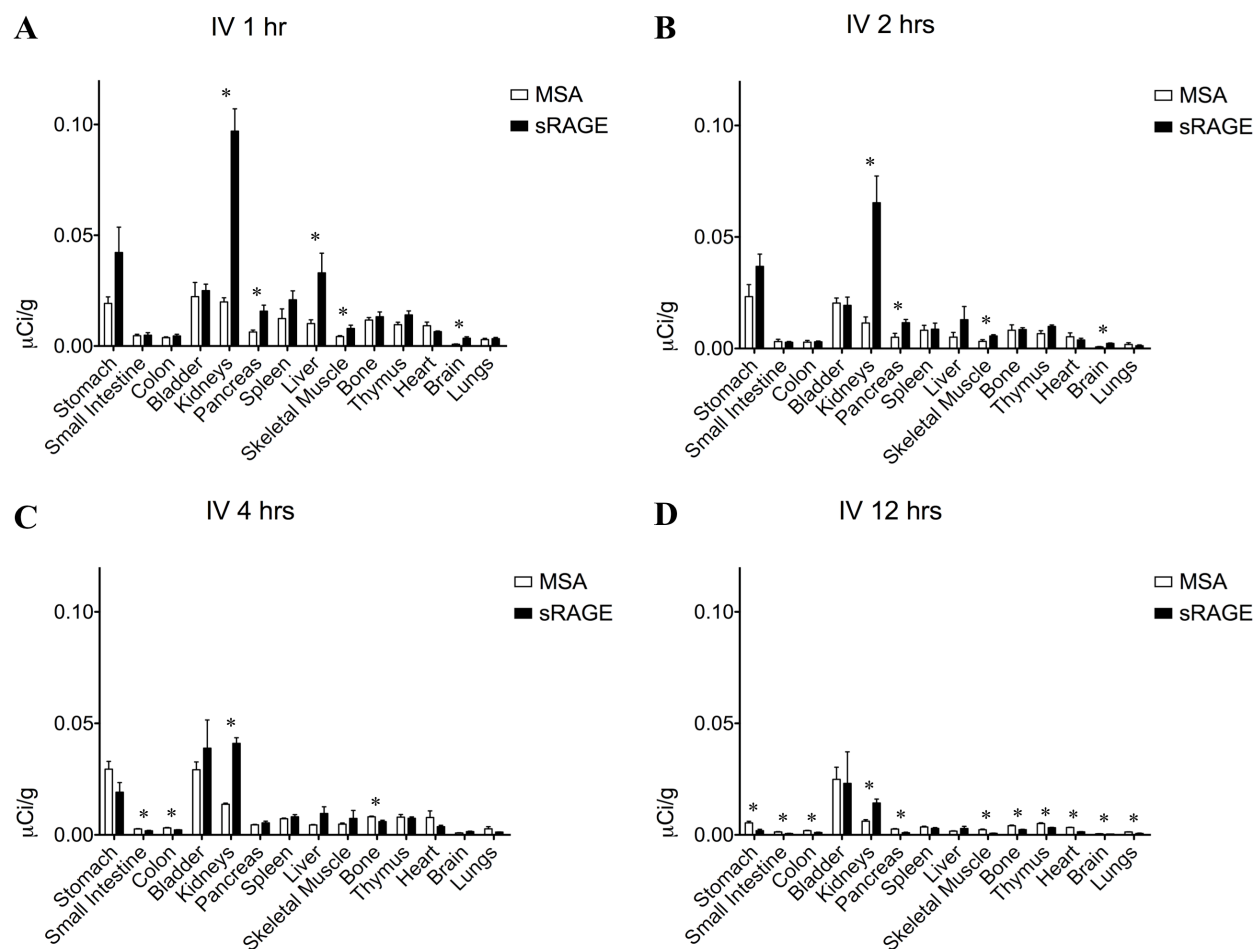


Figure 4. Organ biodistribution of intravenously-administered sRAGE or MSA in mice.

Biodistribution of sRAGE or MSA (A) 1 hour, (B) 2 hours, (C) 4 hours, and (D) 12 hours after i.v. injection.

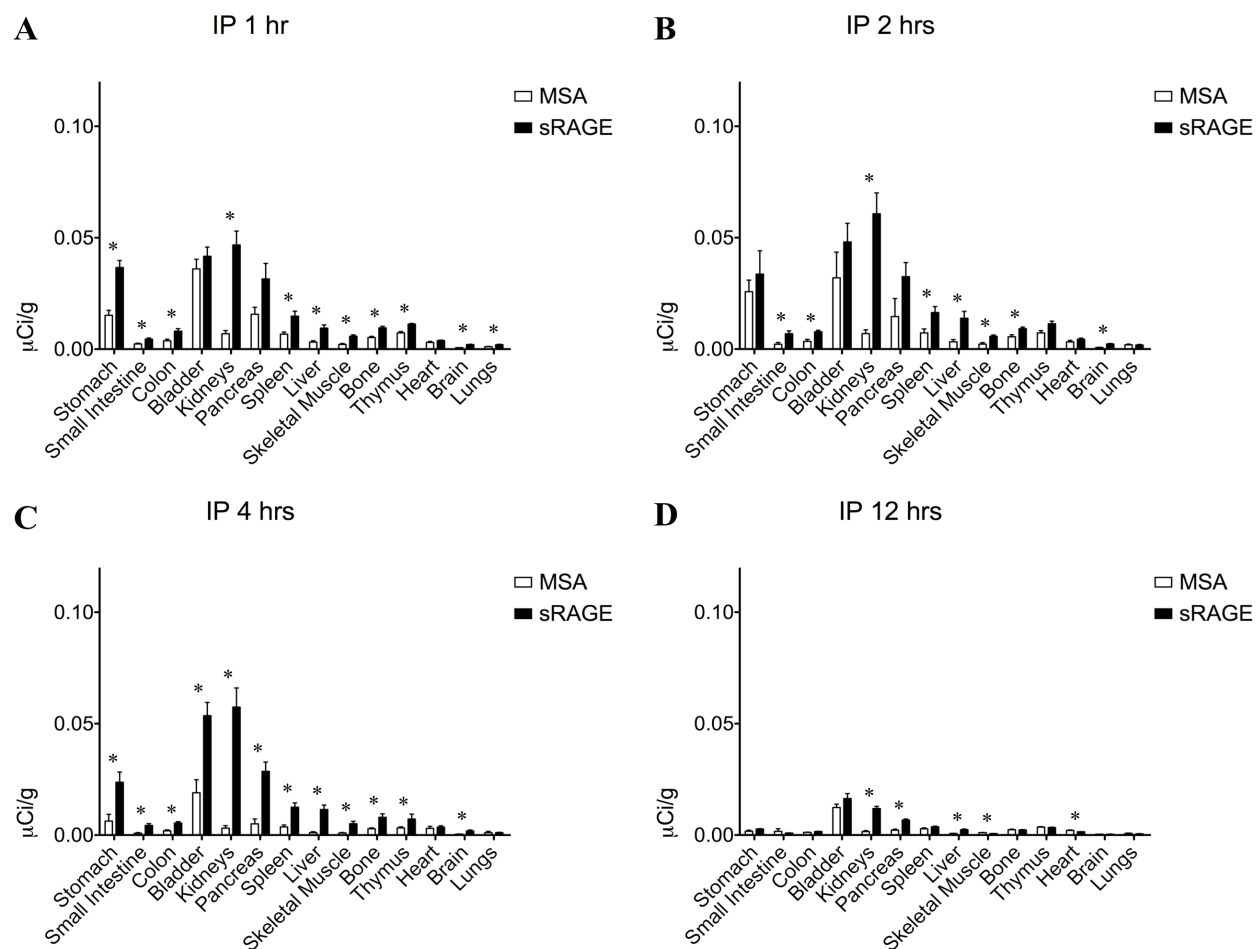


Figure 5. Organ biodistribution of intraperitoneally-administered sRAGE or MSA in mice.
 Biodistribution of sRAGE or MSA (A) 1 hour, (B) 2 hours, (C) 4 hours, and (D) 12 hours after i.p. injection.

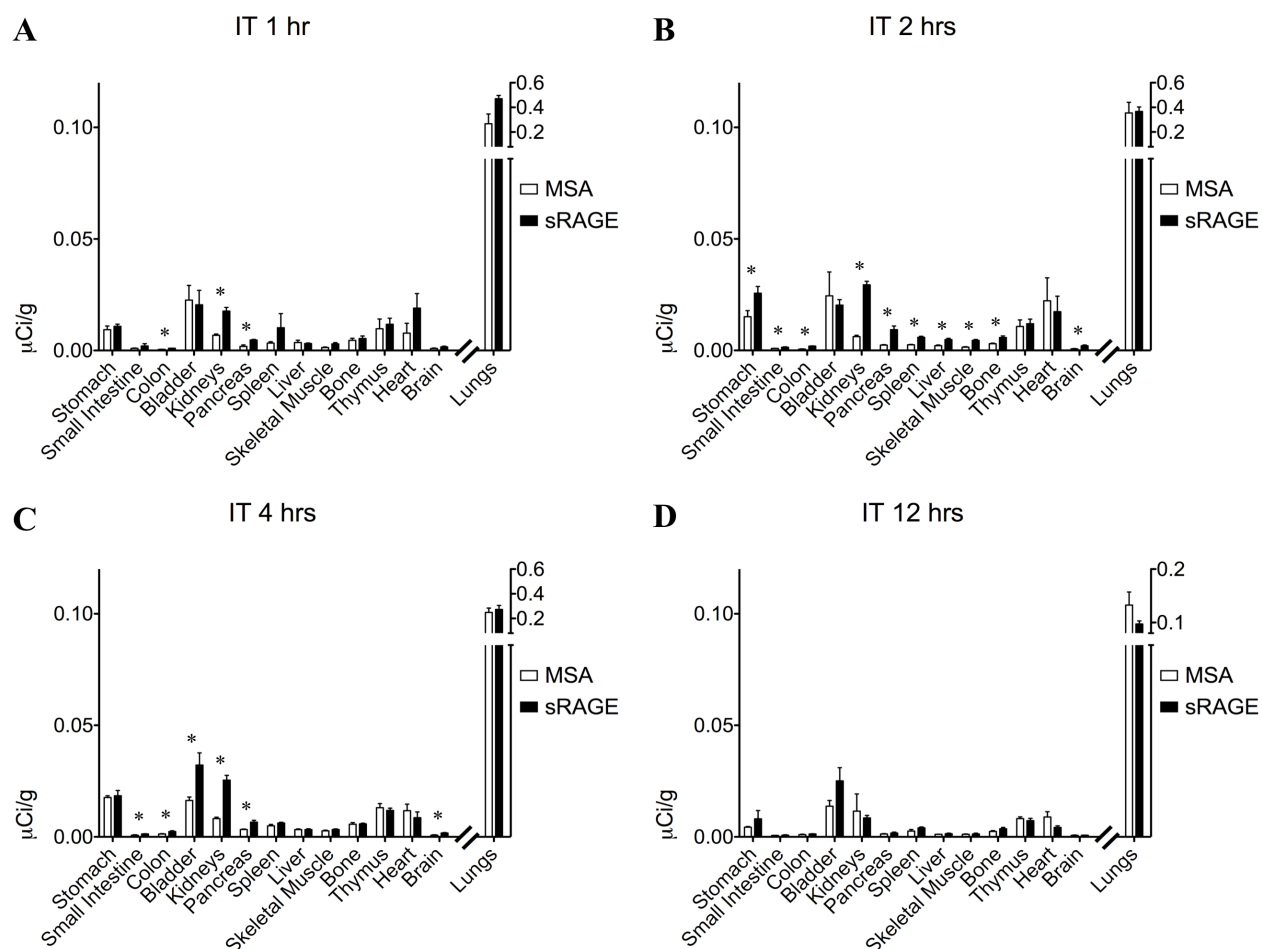


Figure 6. Organ biodistribution of intratracheally-administered sRAGE or MSA in mice.

Biodistribution of sRAGE or MSA (A) 1 hour, (B) 2 hours, (C) 4 hours, and (D) 12 hours after i.t. instillation.

4.2 SOLUBLE RAGE IS RAPIDLY CLEARED FROM THE LUNG, INTACT AND AT THE ALVEOLAR-CAPILLARY INTERFACE

To investigate the kinetics of sRAGE clearance from the lung, the biodistribution data for i.t. administration was plotted with respect to time and mono-exponential decay fitting was performed. Bi-exponential and multi-exponential fitting was not taken into consideration because there is insufficient mechanistic understanding at the microscopic level of the processes by

which either MSA or sRAGE are cleared from lung, and because the mono-exponential fit provides an adequate understanding of the residence time of exogenously-administered MSA and sRAGE in lung. Because the ^{125}I -labeled MSA radioactivity in lung at the 1-hour time point was substantially lower than the radioactivity of ^{125}I -labeled sRAGE in lung at the same time point, it was reasoned that this discrepancy was due to error in the i.t. instillation procedure and the data for MSA at 1 hour following treatment were excluded from the mono-exponential fit. Both MSA and sRAGE were cleared rapidly from the lung, with half-lives of 2.29 and 2.95 hours, respectively (Figure 7A).

Catabolism is one of many possible mechanisms of protein clearance. To test whether sRAGE is removed from the lung by degradation, lung homogenates from animals that had received MSA or sRAGE by i.t. instillation were separated by SDS-PAGE and gel autoradiography was subsequently performed to detect the various radiolabeled species present. Comparison with the gel autoradiograph of the original electrophoretically-separated ^{125}I -labeled purified proteins (Figure 3B) indicates that, while the higher- and lower-molecular weight impurities persist, neither protein undergoes appreciable proteolysis as it is eliminated from normal healthy lung (Figure 7B).

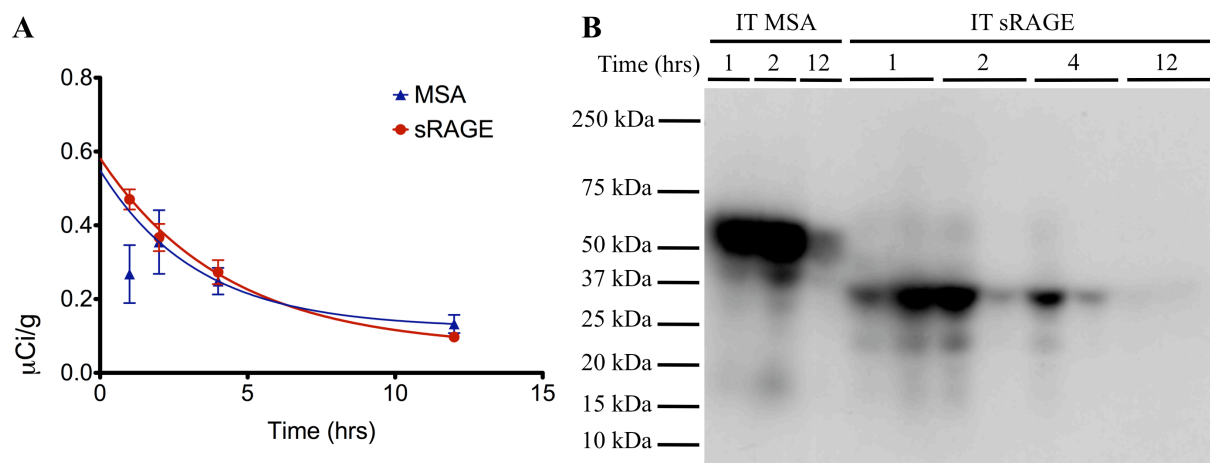


Figure 7. Clearance of intratracheally-administered sRAGE or MSA from the lung is rapid and non-proteolytic.

(A) Exogenous sRAGE or MSA persistence in the lung over time. (B) SDS-PAGE separation and gel autoradiography of lung homogenates from mice given sRAGE or MSA i.t. and sacrificed at the indicated time points thereafter.

To determine the cell and tissue sites at which sRAGE egress from the lung occurs, autoradiography was performed on sections of lung from mice give sRAGE by i.t. instillation. sRAGE (and MSA, results not shown) rapidly reaches the alveolar compartment (there is no apparent predilection for the bronchial epithelium, type II alveolar epithelial cells, or alveolar macrophages) and thence the circulation (Figure 8).

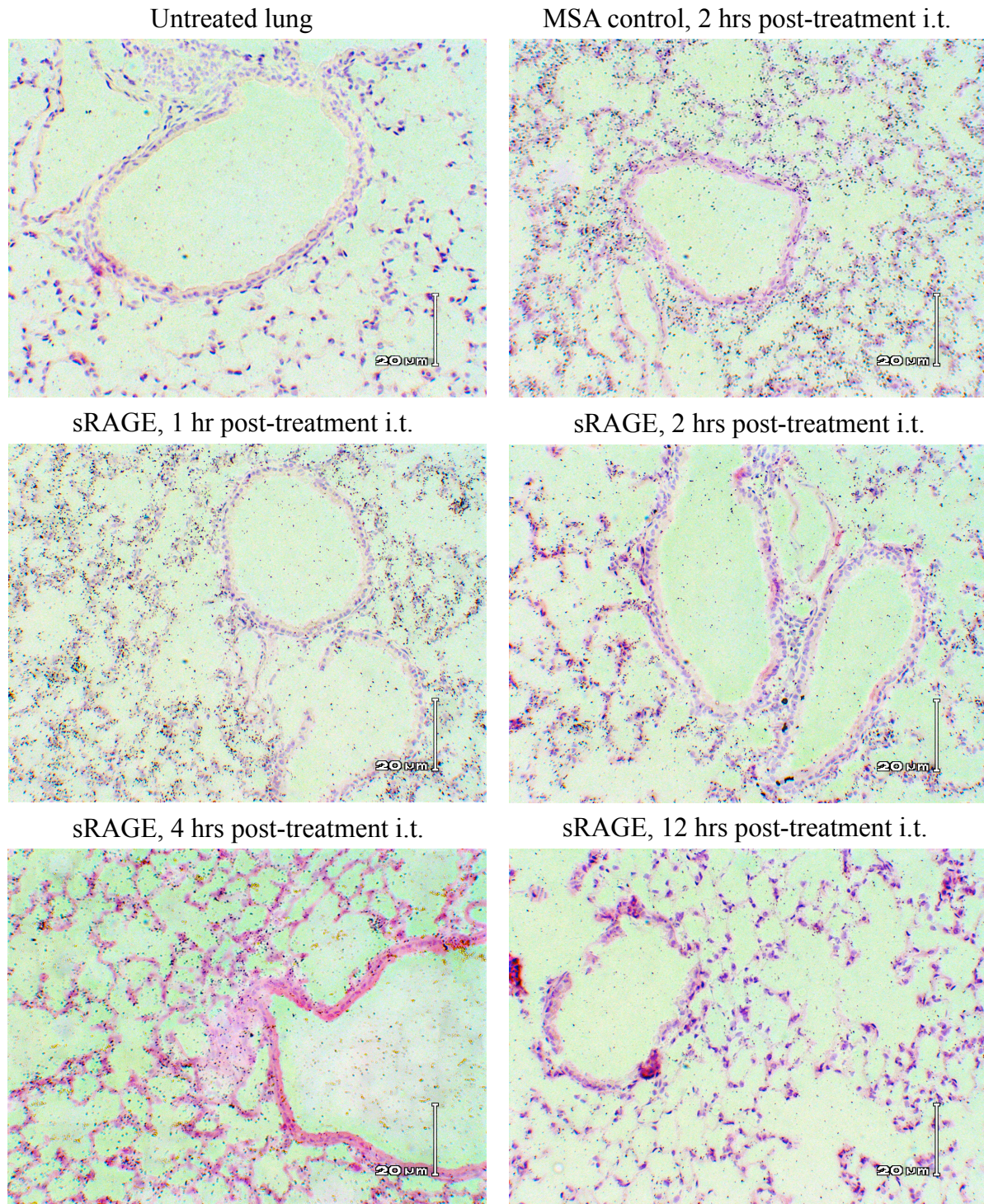


Figure 8. Clearance of intratracheally-administered sRAGE from the lung occurs at the alveolar-capillary interface.

Representative light micrographs/autoradiographs recorded at 20X magnification of sections of lung from mice given sRAGE (or MSA, shown for comparison) i.t. and sacrificed at the indicated time points thereafter. Lung from untreated mice serves as autoradiography background control.

4.3 EXOGENOUS SOLUBLE RAGE DOES NOT RECRUIT EOSINOPHILS TO THE LUNG

To test the capacity of sRAGE to recruit eosinophils, endotoxin-depleted sRAGE or MSA was administered by i.t. instillation and mice were sacrificed at various time points thereafter. Eosinophil infiltration was assessed by immunofluorescence microscopy on lung sections, using collagen IV to discriminate pulmonary architecture and major basic protein (MBP) to detect eosinophils. Although these results were not quantified, no appreciable difference in eosinophil burden in response to MSA vs. sRAGE was apparent. Even when sRAGE was given on two consecutive days, no difference from MSA treatment was observed (Figure 9).

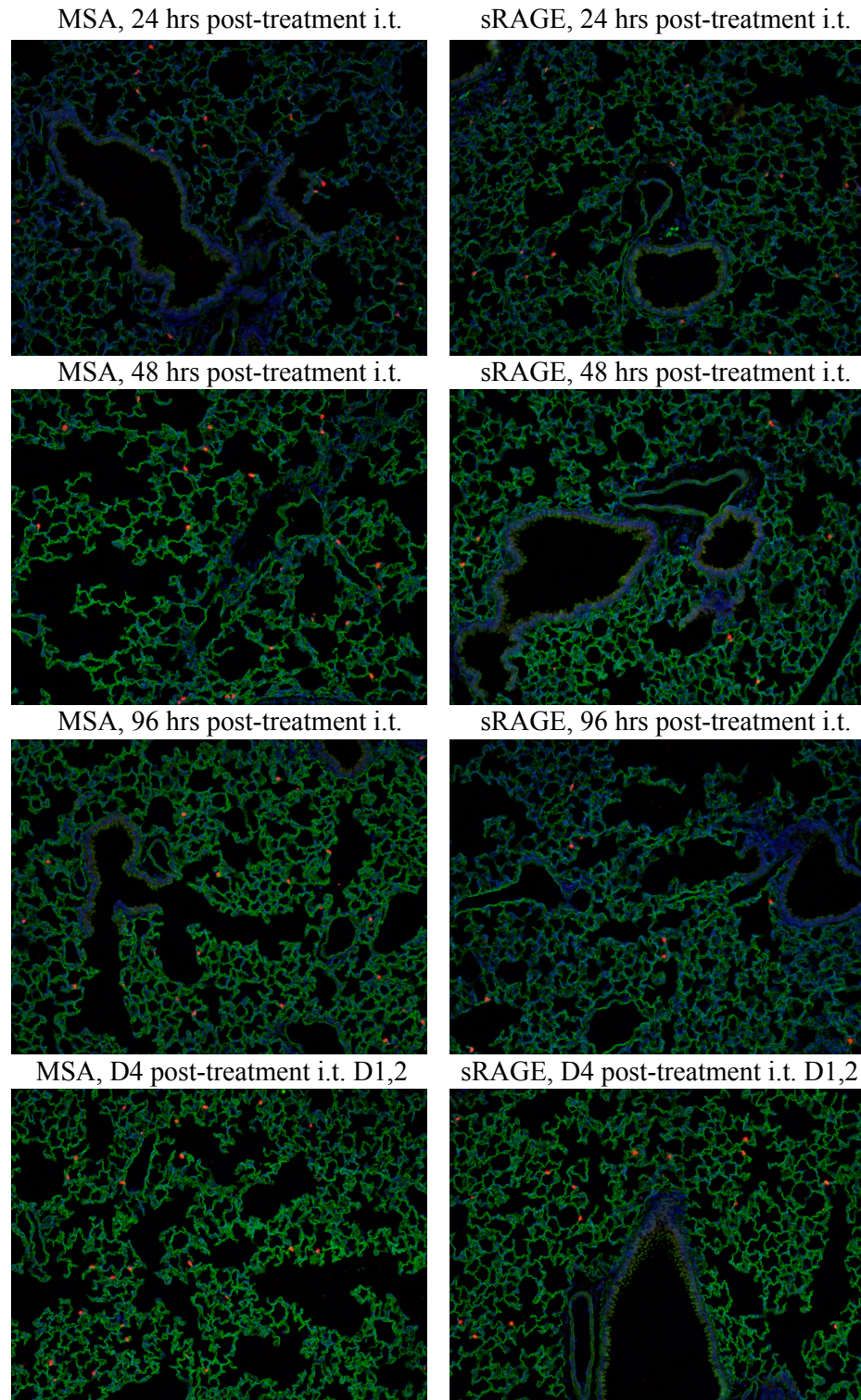


Figure 9. Intratracheally-administered sRAGE does not draw eosinophils into the lung.

Representative immunofluorescence photomicrographs recorded at 10X magnification of sections of lung from mice treated with 50 μ g MSA or 50 μ g sRAGE, then sacrificed at the indicated time points thereafter. *Green* – collagen IV; *red* – MBP; *blue* – nuclei.

4.4 ALLERGIC AIRWAY DISEASE DOES NOT ALTER PULMONARY RAGE EXPRESSION

Dysregulated expression or localization of one or more molecular mediators implicated in a pathogenic process is a common feature of disease. To investigate the regulation of RAGE expression in response to allergens or the allergic disease state itself, mice were subjected to a house dust mite (HDM) model of allergic airway disease/asthma (the protocol outlined in Figure 2A) and assayed for RAGE transcript and protein expression. qRT-PCR indicated a modest down-regulation in RAGE transcript expression in response to HDM extract as compared to saline control (Figure 10A), although this difference was not statistically significant. Nor was any change observed in the total expression level of RAGE protein or the relative proportions of sRAGE as compared to its two membrane-bound isoforms, xRAGE²⁹⁴ and mRAGE (Figure 10B).

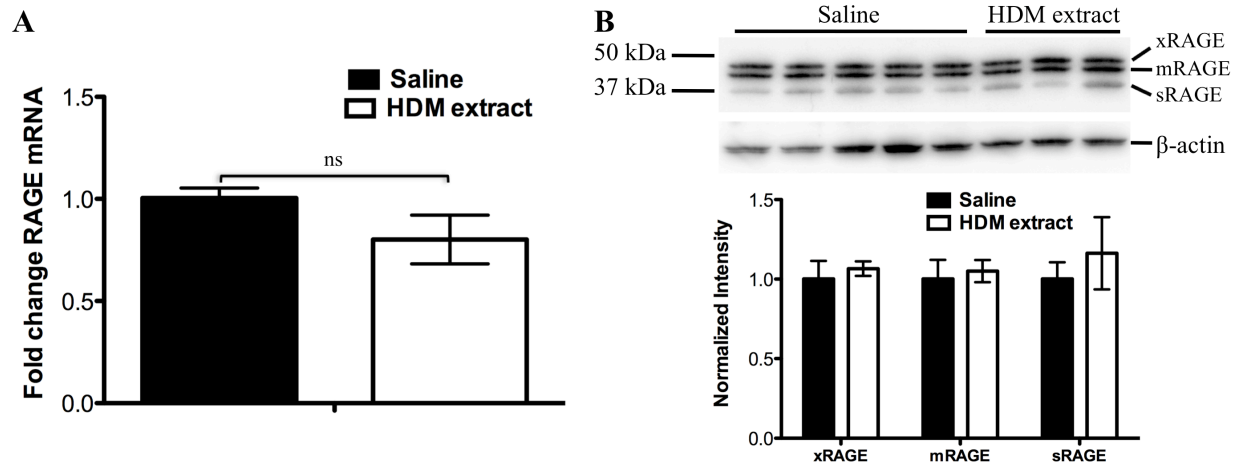


Figure 10. Pulmonary RAGE expression is not altered in an HDM model of allergic airway disease/asthma.

(A) qRT-PCR (GAPDH used as housekeeping control) and (B) immunoblot (β -actin serves as loading control) probing for RAGE, in whole lung homogenates of mice treated intranasally with HDM extract or saline control. Densitometry analysis shows normalized RAGE isoform: β -actin ratios for each strain/treatment group. $n=3-5$ per treatment group.

4.5 ALLERGIC AIRWAY DISEASE DOES NOT INDUCE SHEDDING OF SOLUBLE RAGE

sRAGE shedding has been demonstrated to occur in the context of acute pulmonary injury. Moreover, sRAGE has been found by other investigators to be elevated in the BALF of asthma patients.²⁸⁵ To determine if such shedding into the airspaces or circulation was occurring, BALF (~10% of the total volume recovered from both lungs) and serum from mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A) were assayed by immunoblotting for the presence of sRAGE. sRAGE could be detected in neither BALF nor serum (Figure 11).

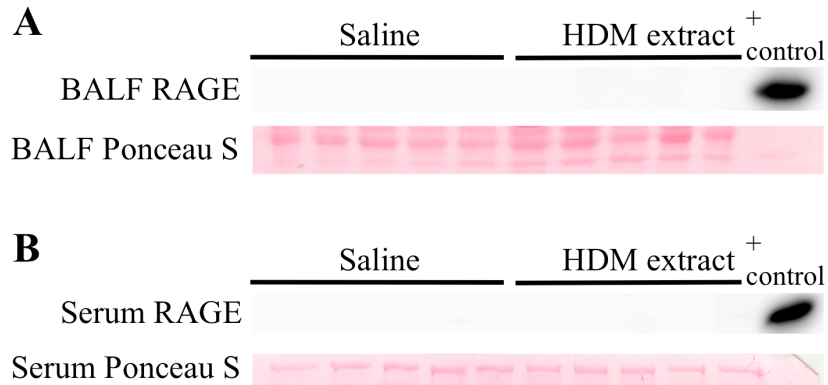


Figure 11. sRAGE is not shed into the airspaces or the circulation in an HDM model of allergic airway disease/asthma.

Immunoblot probing for RAGE in (A) BALF and (B) serum samples of mice treated intranasally with HDM extract or saline control. Ponceau S stain serves as loading control, and purified sRAGE as positive control.

4.6 ALLERGIC AIRWAY DISEASE DOES NOT ALTER PULMONARY RAGE

LOCALIZATION

As RAGE is abundantly expressed by type I alveolar epithelial cells, it was thought that subtle changes in RAGE expression in other cell types more closely associated with allergic airway disease/asthma, such as bronchial epithelial cells or inflammatory cells, might be undetectable by whole lung expression assays. To address this question, immunofluorescence microscopy studies of lung sections from mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A) were performed, using collagen IV to delineate the basement membrane, which approximates the basolateral aspect of RAGE-expressing alveolar epithelium. No appreciable change in bronchial, vascular, or alveolar RAGE expression in response to HDM extract sensitization and challenge could be detected (Figure 12).

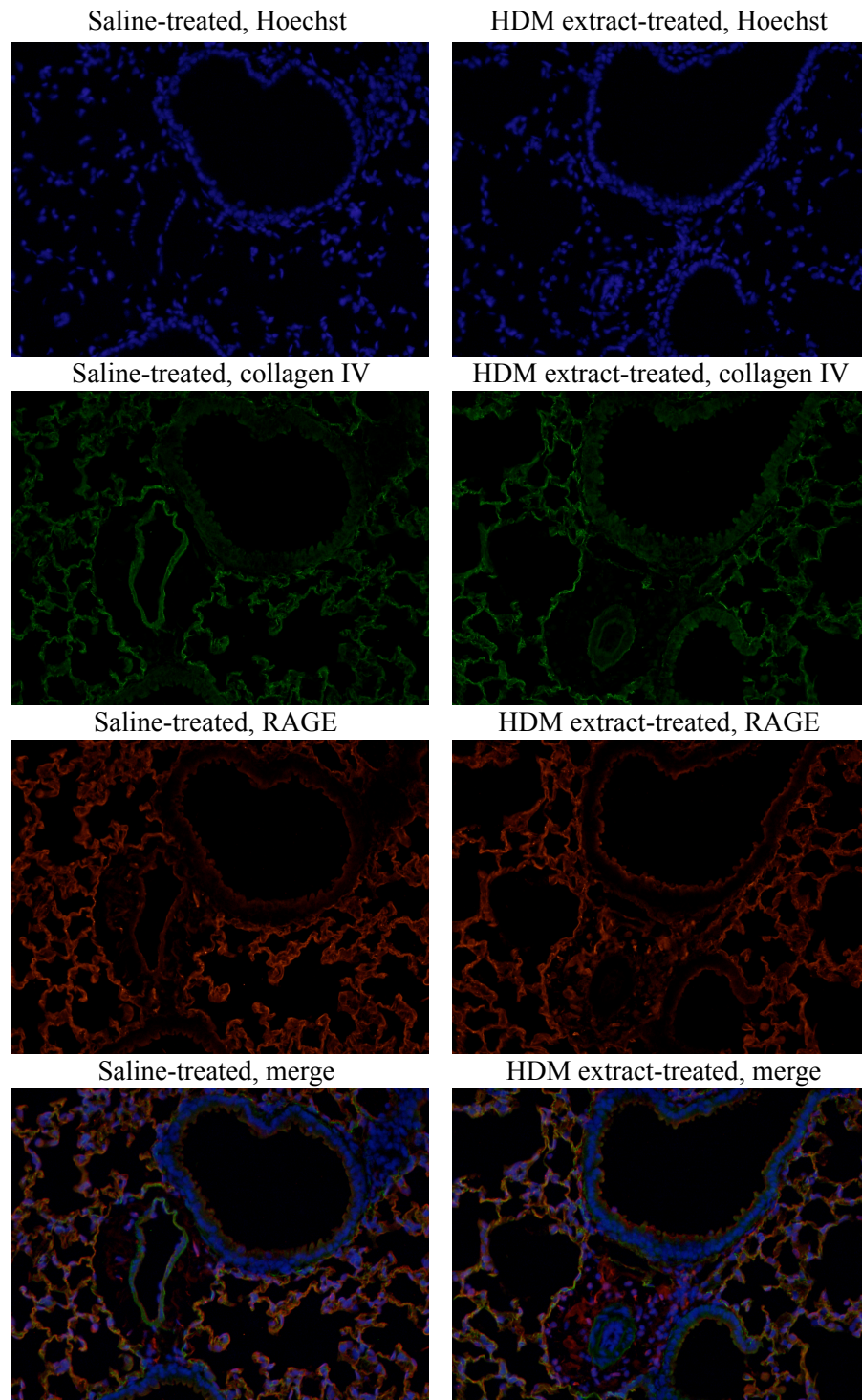


Figure 12. Pulmonary RAGE localization is not altered in an HDM model of allergic airway disease/asthma.

Representative immunofluorescence photomicrographs recorded at 10X magnification of sections of lung from mice treated with HDM extract or saline control. *Green* – collagen IV; *red* – RAGE; *blue* – nuclei; *yellow* – co-localization.

Because asthma is a chronic inflammatory disease and because RAGE has been reported to be expressed on hematopoietic cells, notably monocytes-macrophages,^{34,295,296} eosinophils,⁵⁹ and T cells,^{56,58} lung sections from mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A) were inspected by immunofluorescence microscopy for the expression of RAGE by cells of the hematopoietic compartment. Eosinophils and CD4⁺ helper T cells do not express RAGE (Figure 13A,B), whereas alveolar macrophages appear to express RAGE at low levels at baseline (Figure 13C, middle). RAGE expression appears to co-localize with F4/80 expression at the cell surface in saline controls but has a more punctate and intracellular distribution in lungs of mice treated with HDM extract (Figure 13C, right).

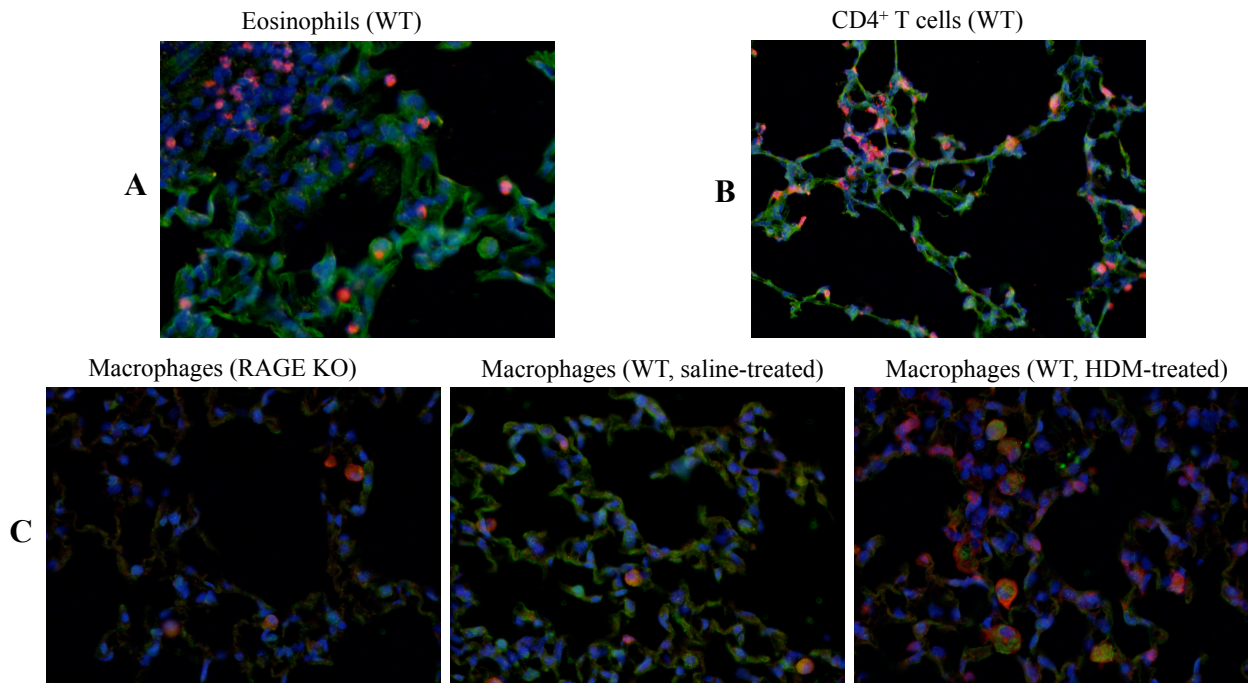


Figure 13. Eosinophils and helper T lymphocytes do not express RAGE, whereas macrophages express low levels of RAGE.

Representative immunofluorescence photomicrographs recorded at 20X magnification of pulmonary (A) eosinophils, (B) CD4⁺ T lymphocytes, or (C) macrophages: left, RAGE KO control; middle, saline-treated lung; right, HDM extract-treated lung. *Green* – RAGE; *red* – MPB (eosinophils), CD4 (helper T lymphocytes), or F4/80 (macrophages); *blue* – nuclei; *yellow* – co-localization.

4.7 IN THE ABSENCE OF RAGE, CHANGES IN PULMONARY FUNCTION PARAMETERS CONSISTENT WITH ASTHMA DO NOT DEVELOP IN RESPONSE TO HDM ALLERGEN

Expression of a pathogenetic mediator need not be altered in a disease state, particularly when the mediator in question is a receptor with multiple, potentially mutually-antagonistic, roles. Therefore, to explore whether the absence of RAGE affects the physiologic response to allergen sensitization and challenge, wild type or RAGE KO mice were subjected to an HDM model of allergic airway disease/asthma (Figure 2A). Pulmonary function testing performed using forced oscillation technique on a flexiVent apparatus demonstrated significant alterations in airway function in wild type mice treated with HDM extract, consistent with an asthmatic profile (Figure 14). These changes in responsiveness to methacholine challenge were evident in the parameters corresponding to central airway resistance (R_n), small airway tissue damping or resistance (G), and tissue elastance (H), respectively. The modest change in R_n (Figure 15A), as compared to what is often seen in studies in BALB/c mice, is consistent with findings of previous studies in the C57BL/6 strain.²⁹⁷ The picture with the R_n parameter is somewhat complex, with a slightly elevated responsiveness to methacholine in naïve RAGE KO mice as compared to wild type counterparts, but no difference between saline-treated versus HDM extract-treated RAGE KO mice is demonstrable. However, it is important to note that RAGE KO mice treated with HDM extract demonstrate G and H parameters (Figure 15B,C) indistinguishable from those of naïve wild type and RAGE KO mice.

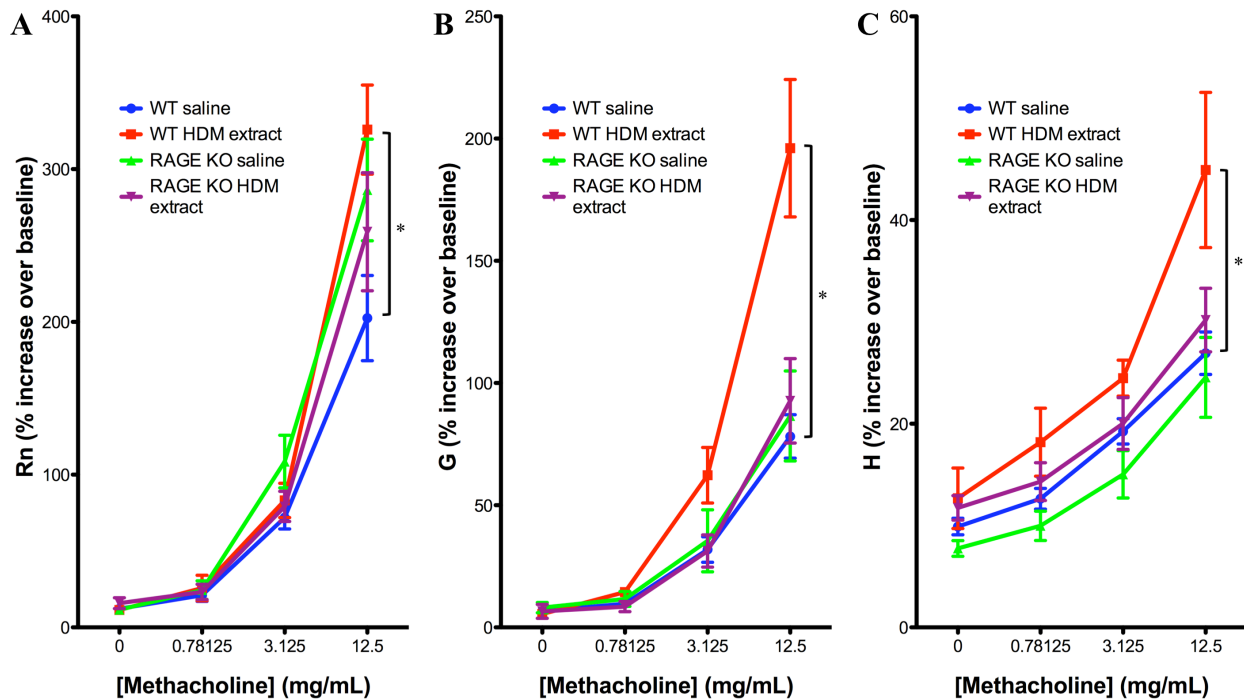


Figure 14. Mice lacking RAGE do not develop airway hypersensitivity to methacholine challenge in response to HDM allergen.

Dose-response curves of parameters of (A) central airway resistance (Rn), (B) tissue damping (G), and (C) tissue elastance (H), in response to methacholine challenge, in mice treated with HDM extract or saline control. n=6-10 per strain/treatment group.

4.8 IN THE ABSENCE OF RAGE, HDM ALLERGEN DOES NOT EFFECT PULMONARY EOSINOPHILIA

There is a prominent eosinophilic inflammatory component in allergic airway disease/asthma, and to assess this aspect of disease wild type and RAGE KO mice were subjected to an HDM model of allergic airway disease/asthma (Figure 2A). Modified Romanowsky staining of BALF cells illustrated markedly elevated cell counts and eosinophilia in the allergic wild type mice compared to saline-treated controls (Figure 15).

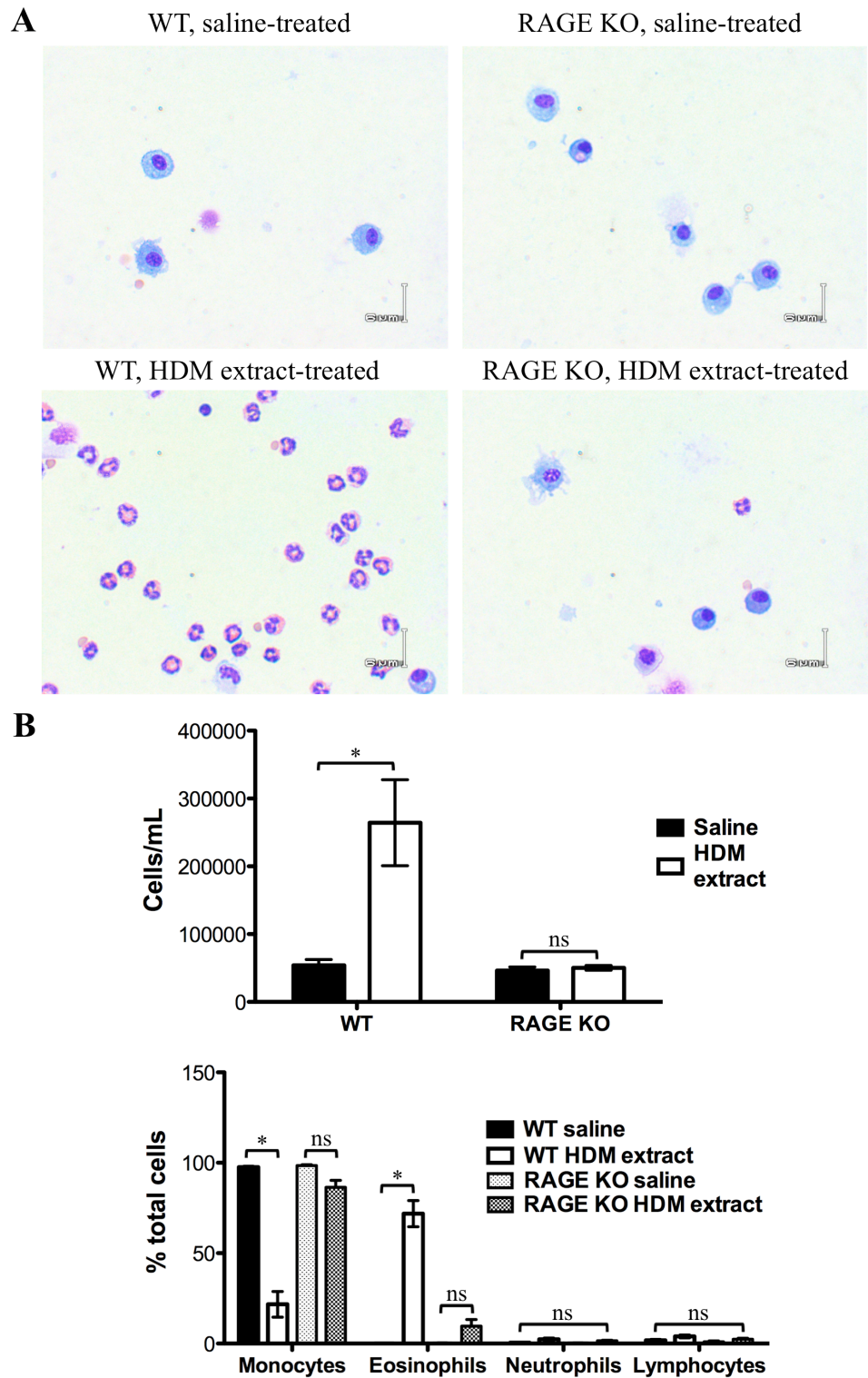


Figure 15. Mice lacking RAGE do not develop airway eosinophilia in response to HDM allergen.

(A) Representative photomicrographs recorded at 40X magnification and (B) graphical summaries of cell counts and differentials, of Romanowsky-stained cytopins prepared from BALF from mice treated with HDM extract or saline control. n=6-10 per strain/treatment group.

Histologic evaluation of lung sections by H&E stain revealed peribronchial, perivascular, and interstitial eosinophilia in lungs of allergic wild type mice but not in controls (Figure 16). In contrast, RAGE KO mice sensitized and challenged with HDM extract had essentially no inflammatory infiltrates, suggesting that RAGE has an important role in drawing eosinophils to the lung in response to HDM allergen.

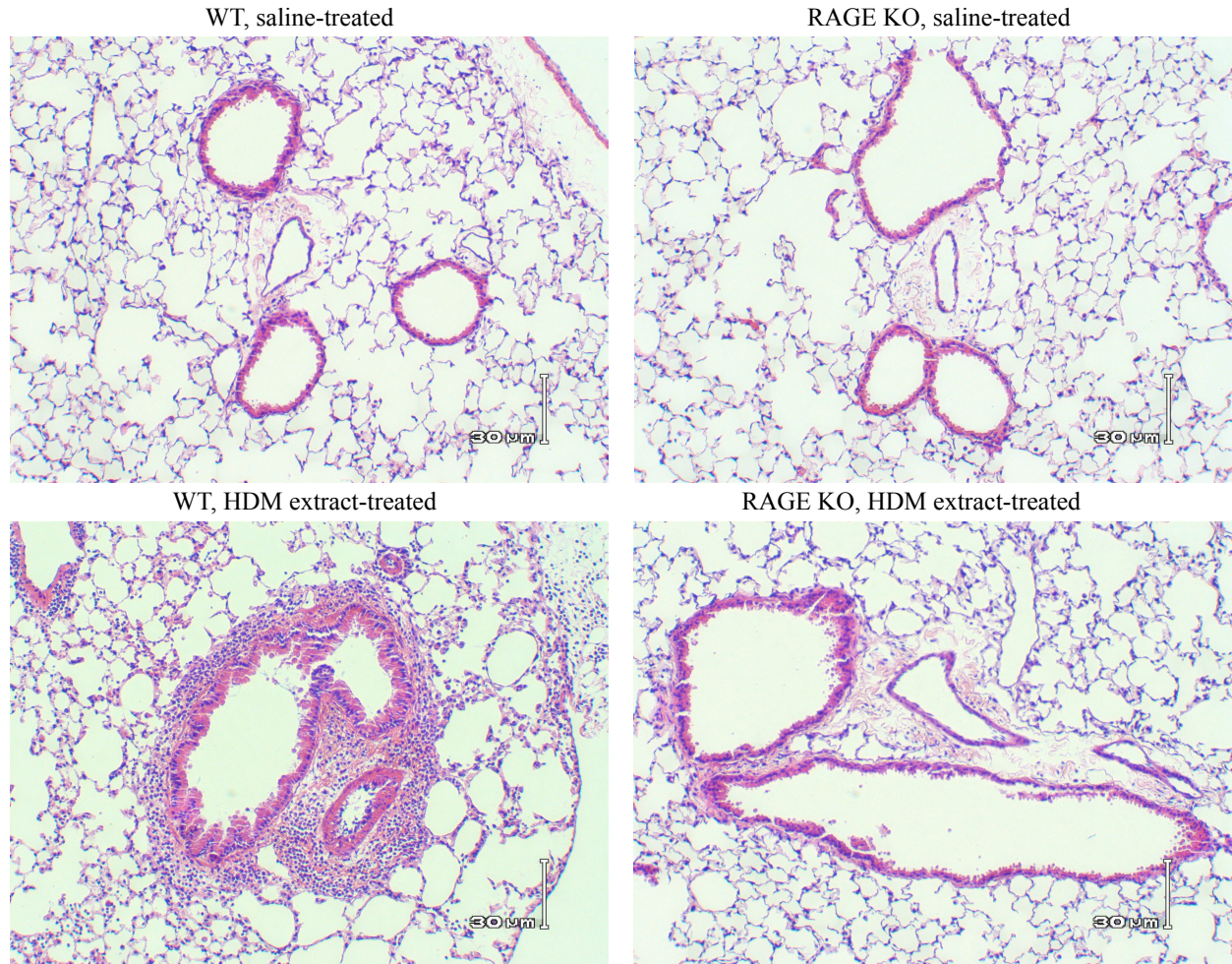


Figure 16. Mice lacking RAGE do not develop peribronchial, perivascular, and interstitial eosinophilia in response to HDM allergen.

Representative photomicrographs recorded at 20X magnification off H&E-stained lung sections from mice treated with HDM extract or saline control. n=6-10 per strain/treatment group.

4.9 IN THE ABSENCE OF RAGE, HDM ALLERGEN DOES NOT INDUCE MUCUS HYPERSECRETION

Mucus hypersecretion is a hallmark feature of asthma. To assess for goblet cell hyperplasia, lung sections from wild type or RAGE KO mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A) were evaluated for expression of mucin by periodic acid-Schiff (PAS) stain. As anticipated, wild type mice showed goblet cell hyperplasia and elevated expression of mucin in numerous bronchi of the allergic wild type mice compared to saline-treated controls (Figure 17). However, RAGE KO mice did not demonstrate enhanced mucus expression when treated with HDM extract, suggesting that RAGE is involved in key changes in airway structure and function characteristic of allergic airway disease/asthma.

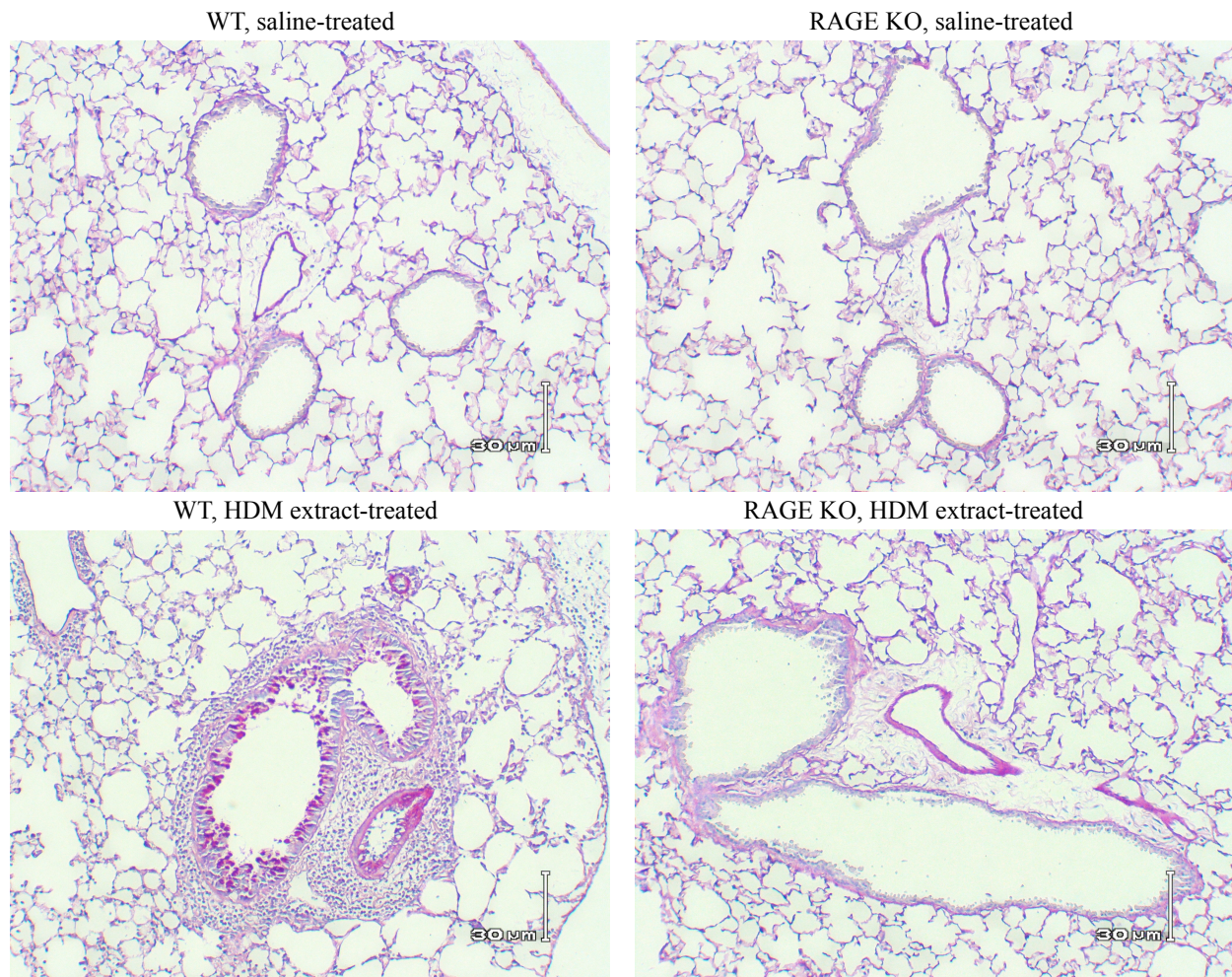


Figure 17. Mice lacking RAGE do not develop elevated mucin expression in response to HDM allergen.

Representative photomicrographs recorded at 20X magnification of PAS-stained lung sections from mice treated with HDM extract or saline control. n=6-10 per strain/treatment group.

4.10 ENDOGENOUS RAGE BINDING PARTNERS ARE DIFFERENTIALLY EXPRESSED IN RESPONSE TO ALLERGIC AIRWAY DISEASE

A key question arising from studies of the role of RAGE in an HDM model of allergic airway disease/asthma concerns the identity of the RAGE ligand(s) mediating the pro-allergic function

of RAGE. This question was addressed both by studying an ovalbumin model of allergic airway disease/asthma, as well as the direct approach of probing for RAGE binding partners. HDM extract was passed over an sRAGE-conjugated resin under gentle low-salt conditions, and then adsorbed binding partners were eluted by increasing the salt concentration. The eluted proteins were concentrated, separated by electrophoresis and visualization was attempted using Coomassie Brilliant Blue stain (Figure 18A), which has a detection limit of ~50 ng. Because no bands could be detected on this attempt, the entire experiment was repeated, with visualization achieved using SYPRO Ruby gel stain (Figure 18B), which has a detection limit of below 1 ng. There is therefore a >100,000-fold difference between the estimated quantity of HDM extract protein passed over the column (~5 mg) and the minimum quantity detectable by Coomassie Brilliant blue stain (~50 ng). Despite the fact that such scarce binding partners are of dubious biological relevance, the eluted proteins were submitted for identification by mass spectrometry. The spectrum of RAGE-binding HDM extract proteins (Table 1, appendix) is unremarkable (as no species-restriction to *Dermatophagoides pteronyssinus* was possible in the peptide matching database, homologs in other arthropod species are erroneously identified as matches), and none have been suggested to have particular relevance to the development of allergic airway disease/asthma in mice or humans.

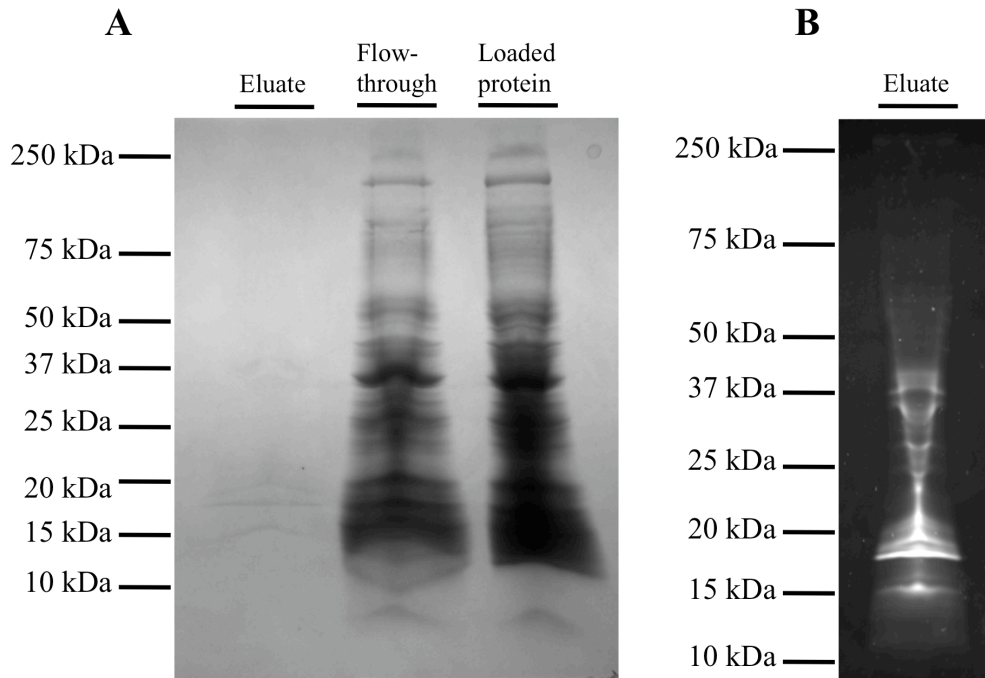


Figure 18. RAGE binding partners are scarce in HDM extract.

SDS-PAGE separation and (A) Coomassie Brilliant Blue gel staining or (B) SYPRO Ruby gel staining, of whole HDM extract proteins, non-adsorbed (“flow-through”) HDM extract proteins from sRAGE affinity chromatography, and eluate containing sRAGE-binding proteins in HDM extract

As HDM extract did not prove to be a major source of RAGE binding partners, and those identified are not particularly compelling, an alternative mechanism was considered: endogenous RAGE ligands, elaborated by one or more unknown cellular players in response to exogenous allergen stimulus, drive RAGE signaling and thereby allergic disease. To explore this possibility, equal quantities of lung homogenate protein from mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A) were passed over an sRAGE-conjugated matrix; the matrix was washed and ligands eluted in a high-salt buffer and concentrated. SDS-PAGE and SYPRO Ruby gel staining demonstrated numerous RAGE-interacting proteins in lung homogenates from all strain/treatment groups. Differential regulation (primarily up-regulation, but also down-regulation) of RAGE binding partners in wild type mice in response to HDM allergen, as

compared to saline-treated controls, was readily apparent (Figure 19). The same expression pattern was not seen in RAGE KO mice treated with HDM extract, which is perhaps indicative of a feed-forward mechanism in which early RAGE ligands act via RAGE to induce the enhanced expression of the additional RAGE ligands. Mass spectrometry identified numerous RAGE-binding proteins (Table 2, appendix), a number of which (e.g. decorin and biglycan) have been reported to have significance in asthma.²⁹⁸⁻³⁰¹ It is also notable that HMGB1 is not among the RAGE ligands identified in any of the lung homogenates. Semi-quantitative analysis was performed to compare HDM extract-treated versus saline control-treated wild type or RAGE KO mice (comparison of treatments within individual strains, but not between strains). A summary of those RAGE binding partners for which the total-protein-normalized emPAI value (defined previously) is greater in the HDM extract-treated group than in the saline control-treated group in at least one of the strains, is given (Table 3, appendix). This gives a rough estimate of which RAGE-interacting proteins demonstrate increased expression in response to HDM extract treatment.

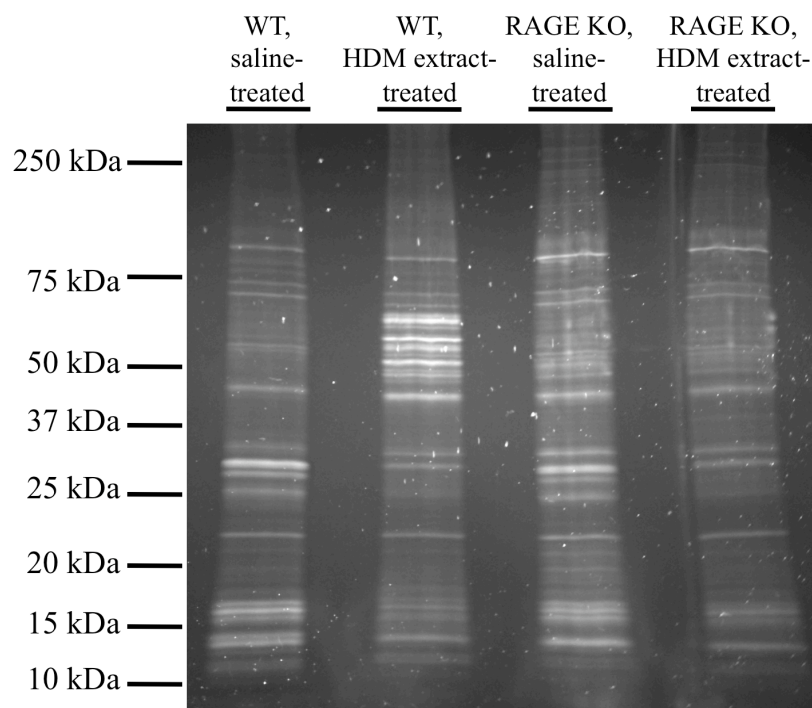


Figure 19. RAGE binding partners are differentially expressed in response to HDM allergen.

SDS-PAGE separation and SYPRO Ruby gel staining of RAGE binding partners isolated by sRAGE affinity chromatography from lung homogenates of wild type and RAGE KO mice subjected to an HDM model of allergic airway disease/asthma.

4.11 IN THE ABSENCE OF RAGE, CHANGES IN PULMONARY FUNCTION PARAMETERS CONSISTENT WITH ASTHMA DO NOT DEVELOP IN RESPONSE TO OVALBUMIN ALLERGEN

To explore the relevance of antigen to the role of RAGE in allergic airway disease/asthma, an ovalbumin model (Figure 2B) was used for comparison to the HDM model. Sensitization differs markedly between the HDM and OVA models, both with respect to location (lung and mediastinal lymph nodes vs. mesentery, respectively) and allergen structure (a blend of arthropod proteins, some with protease activity vs. a chicken egg protein adsorbed to aluminum

hydroxide). Pulmonary function testing indicated alterations in the Rn, G, and H parameters in wild type mice treated with ovalbumin as compared to those treated with saline (Figure 20), analogously to findings in the HDM model. While the change in the Rn parameter did not reach statistical significance, the changes in the G and H parameters did.

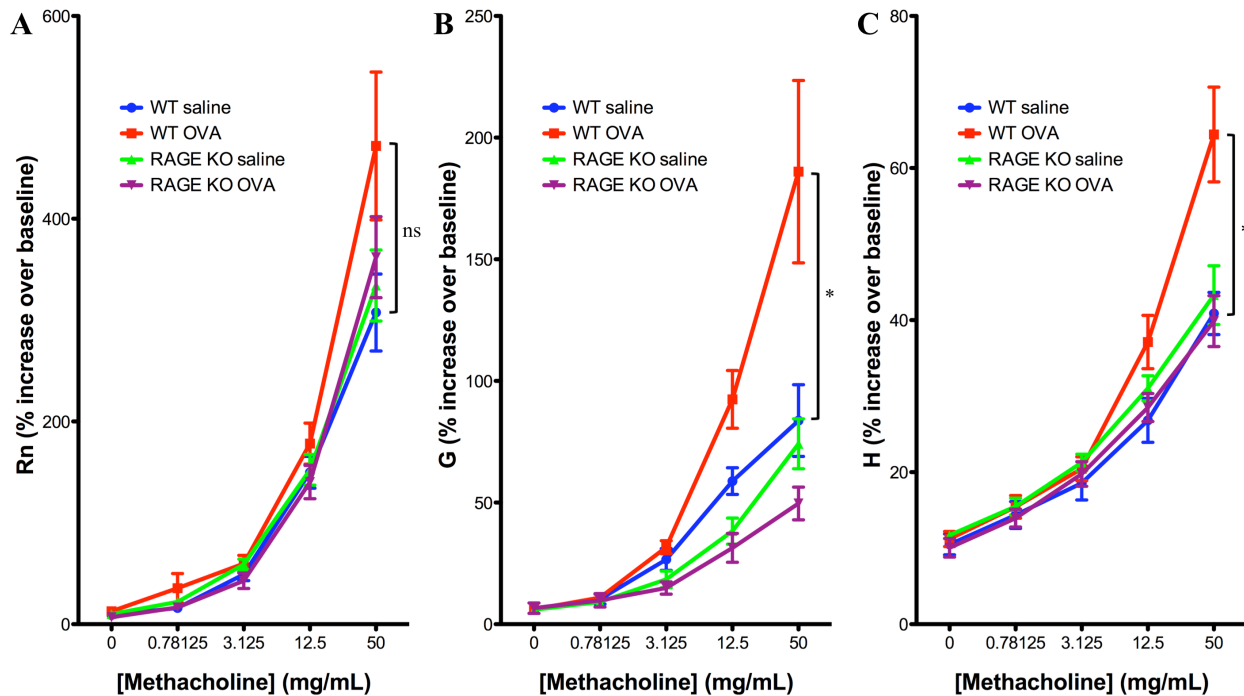


Figure 20. Mice lacking RAGE do not develop airway hypersensitivity to methacholine challenge in response to ovalbumin allergen.

Dose-response curves of parameters of (A) central airway resistance (Rn), (B) tissue damping (G), and (C) tissue elastance (H), in response to methacholine challenge, in mice treated with ovalbumin or saline control. n=8-10 per strain/treatment group.

4.12 IN THE ABSENCE OF RAGE, OVALBUMIN ALLERGEN DOES NOT EFFECT PULMONARY EOSINOPHILIA

To assess for eosinophilic inflammation in an OVA model of allergic airway disease/asthma (Figure 2B), modified Romanowsky staining of BALF cells was performed, illustrating

markedly elevated cell counts and eosinophilia in the allergic wild type mice compared to saline-treated controls (Figure 21). Peribronchial, perivascular, and interstitial eosinophilic inflammation, as assessed by H&E stain, was evident (Figure 22). In contrast, RAGE KO mice sensitized and challenged with ovalbumin had essentially no inflammatory infiltrates, suggesting that the RAGE effect on eosinophil recruitment to the lung observed in an HDM model maintains in the ovalbumin model of allergic airway disease/asthma.

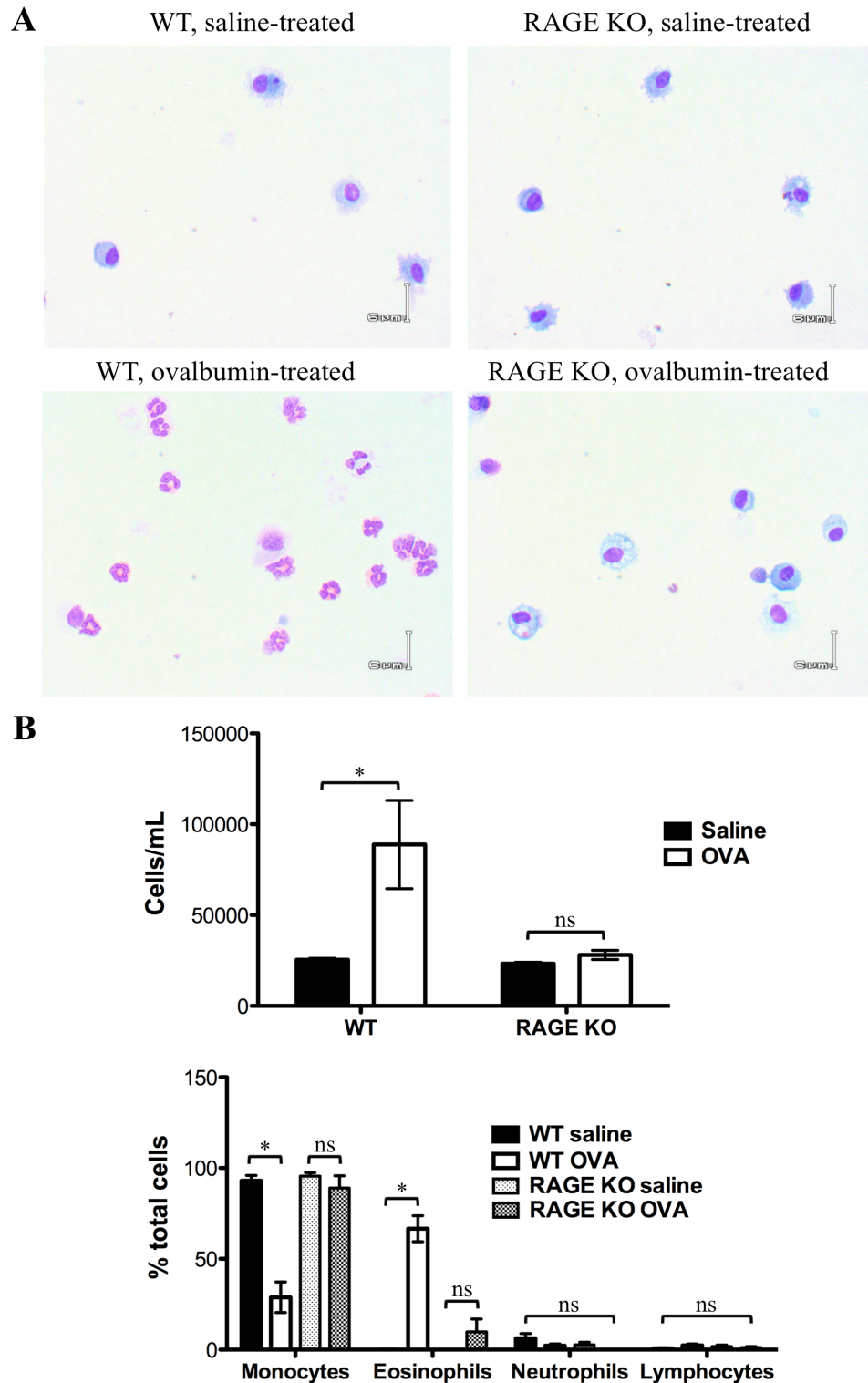


Figure 21. Mice lacking RAGE do not develop airway eosinophilia in response to ovalbumin allergen.
 (A) Representative photomicrographs recorded at 40X magnification and (B) graphical summaries of cell counts and differentials, of Romanowsky-stained cytopins prepared from BALF from mice treated with ovalbumin or saline control. n=8-10 per strain/treatment group.

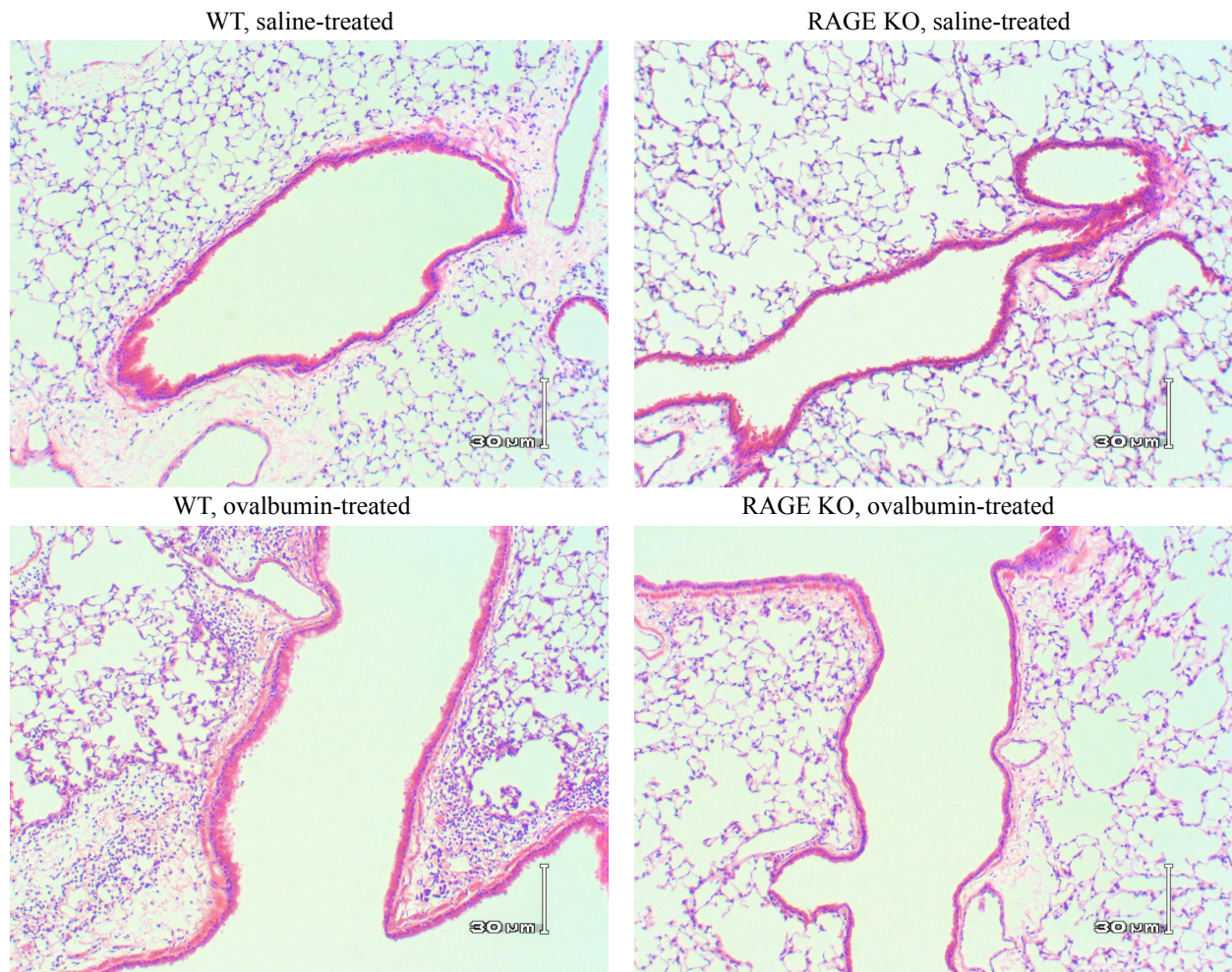


Figure 22. Mice lacking RAGE do not develop peribronchial, perivascular, and interstitial eosinophilia in response to ovalbumin allergen.

Representative photomicrographs recorded at 20X magnification of H&E-stained lung sections from mice treated with ovalbumin extract or saline control. n=8-10 per strain/treatment group.

4.13 IN THE ABSENCE OF RAGE, OVALBUMIN ALLERGEN DOES NOT INDUCE MUCUS HYPERSECRETION

Lung sections from wild type or RAGE KO mice subjected to an OVA model of allergic airway disease/asthma (Figure 2B) were evaluated for expression of mucin by PAS stain. As anticipated,

wild type mice showed goblet cell hyperplasia and elevated expression of mucin in numerous bronchi of the allergic wild type mice compared to saline-treated controls (Figure 23). However, RAGE KO mice did not demonstrate enhanced mucus expression when treated with ovalbumin, suggesting that the RAGE effect on allergic up-regulation of bronchial mucin observed in an HDM model maintains in the ovalbumin model of allergic airway disease/asthma.

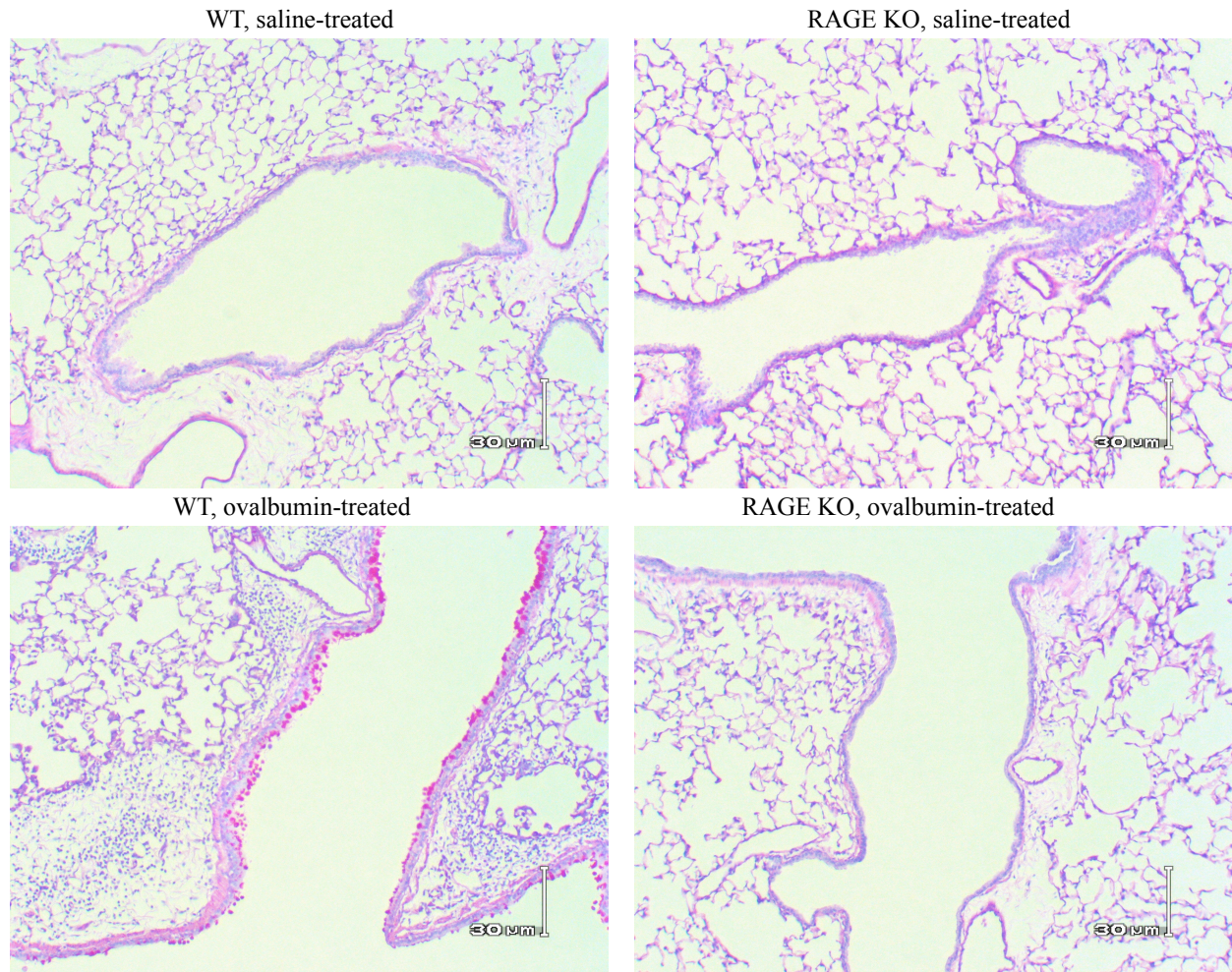


Figure 23. Mice lacking RAGE do not develop elevated mucin expression in response to ovalbumin allergen.

Representative photomicrographs recorded at 20X magnification of PAS-stained lung sections from mice treated with ovalbumin or saline control. n=8-10 per strain/treatment group.

4.14 EXOGENOUS SOLUBLE RAGE HAS NO EFFECT ON HDM ALLERGEN-INDUCED CHANGES IN PULMONARY FUNCTION PARAMETERS

Numerous studies of RAGE in animal models of inflammatory disease have suggested that sRAGE has an anti-inflammatory and generally salutary effect, likely by virtue of its sequestration of RAGE ligands away from the pro-inflammatory membrane isoform.^{82,302,303} To determine which of the two isoforms - mRAGE or sRAGE - is driving the allergic process in the HDM murine model of allergic airway disease/asthma, and to explore whether sRAGE has a therapeutic effect in this disease model, sRAGE (Figure 24) was co-administered i.n. alongside HDM extract; MSA served as a protein control for sRAGE (the protocol is outlined in Figure 2C).

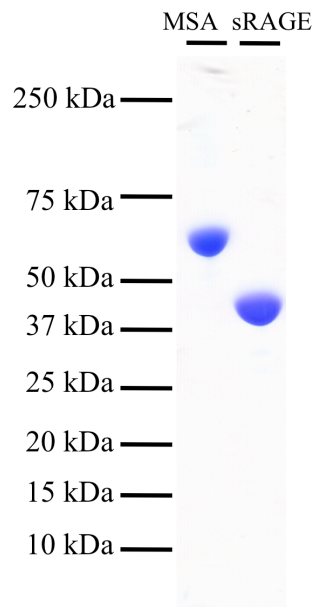


Figure 24. sRAGE and MSA co-administered with HDM allergen are relatively pure.

SDS-PAGE separation and Coomassie Brilliant Blue staining of ~5 µg of MSA or sRAGE preparations used for i.n. administration alone or alongside HDM extract.

Pulmonary function testing did not demonstrate significant differences in parameters Rn, G, or H in mice co-administered sRAGE alongside HDM extract, as compared to those co-administered MSA alongside HDM extract or those receiving HDM extract alone (Figure 25).

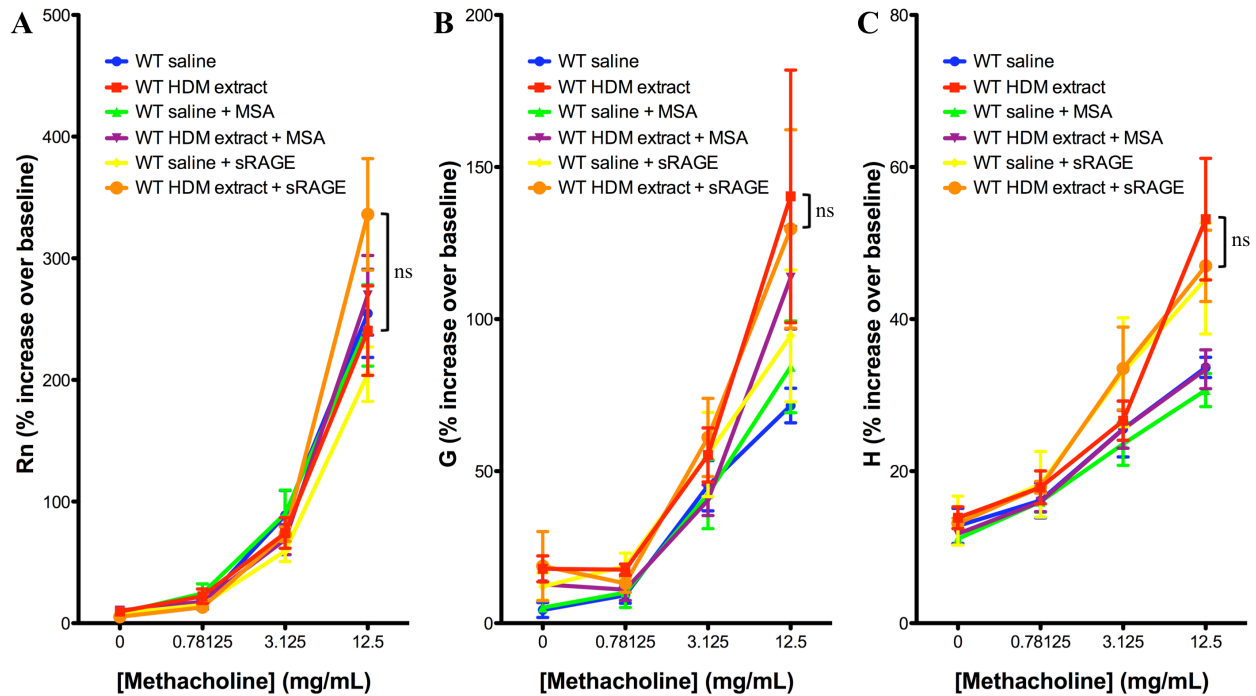


Figure 25. Exogenous sRAGE does not ameliorate airway hypersensitivity to methacholine challenge in response to HDM allergen.

Dose-response curves of parameters of (A) central airway resistance (Rn), (B) tissue damping (G), and (C) tissue elastance (H), in response to methacholine challenge, in mice treated with HDM extract or saline control, alongside sRAGE, MSA, or no additional protein. n=3-4 per treatment group.

4.15 EXOGENOUS SOLUBLE RAGE ATTENUATES PULMONARY EOSINOPHILIC INFLAMMATION ELICITED BY HDM ALLERGEN

Because of the possibility that changes in pulmonary function parameters occur at a low threshold of eosinophil density (thus masking any sRAGE effect on the underlying inflammation (sensitization/challenge protocol outlined in Figure 2C)), modified Romanowsky staining of

BALF cells was performed, illustrating markedly reduced total cell counts and percentage eosinophils in mice co-administered sRAGE alongside HDM extract as compared to either the HDM extract alone or HDM extract alongside MSA (Figure 26). Hematoxylin and eosin stained lung sections (Figure 27A) were evaluated by a pathologist blinded to treatment group for the total number of bronchovascular bundles, the number of bundles demonstrating inflammatory infiltrates, and for scoring of the severity of the inflammation in each bundle. The percentage of bronchovascular bundles involving inflammation and the overall average inflammation severity score were both significantly reduced in mice given sRAGE alongside HDM extract as compared to the two other HDM extract-sensitized/challenged groups (MSA or no protein treatment), mirroring the BALF cell count and differential data (Figure 27B).

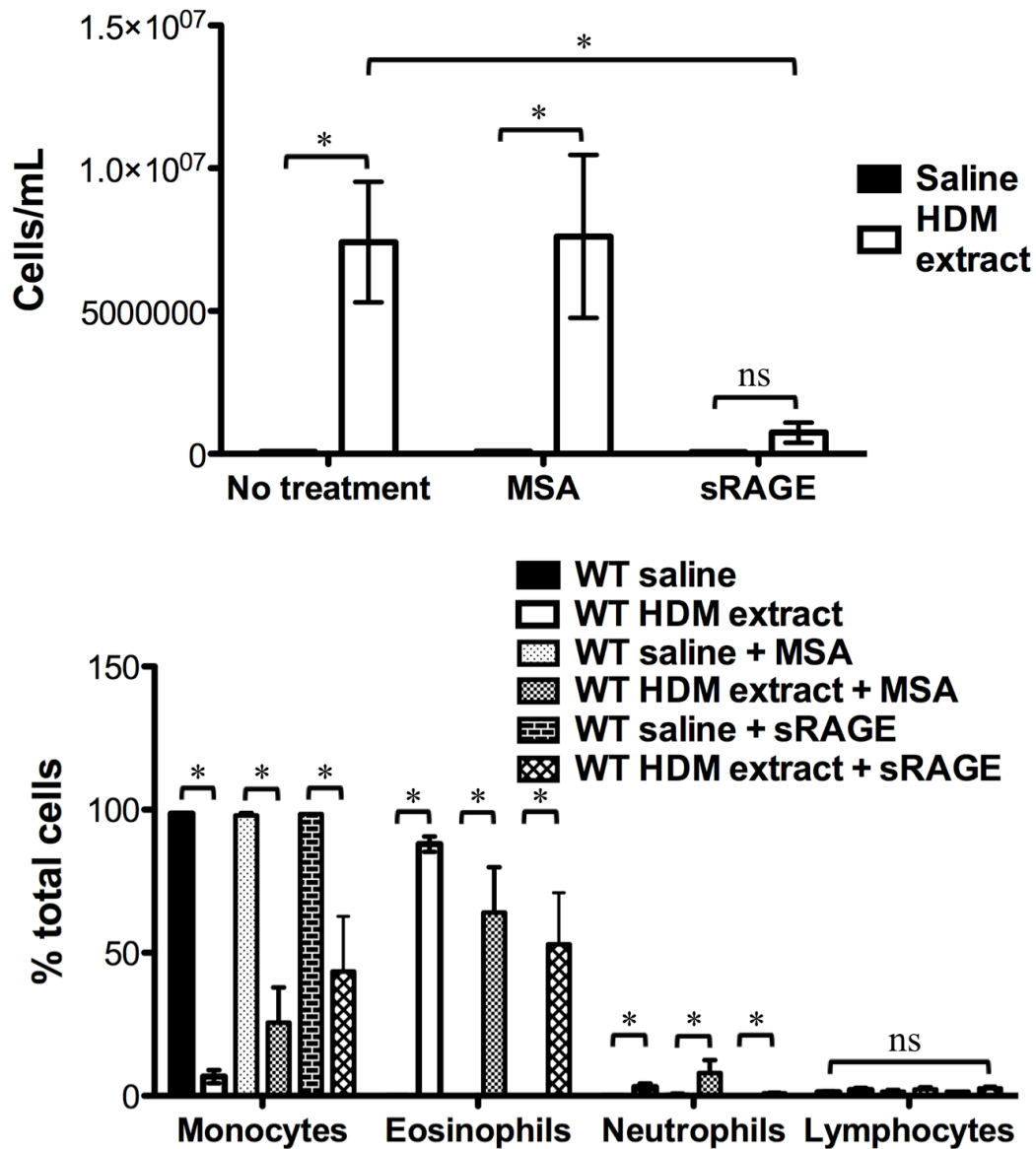


Figure 26. Exogenous sRAGE markedly attenuates airway eosinophilia.

Graphical summaries of cell counts and differentials, of Romanowsky-stained cytopins prepared from BALF from mice treated with HDM extract or saline control, alongside sRAGE, MSA, or no additional protein. n=3-4 per treatment group.

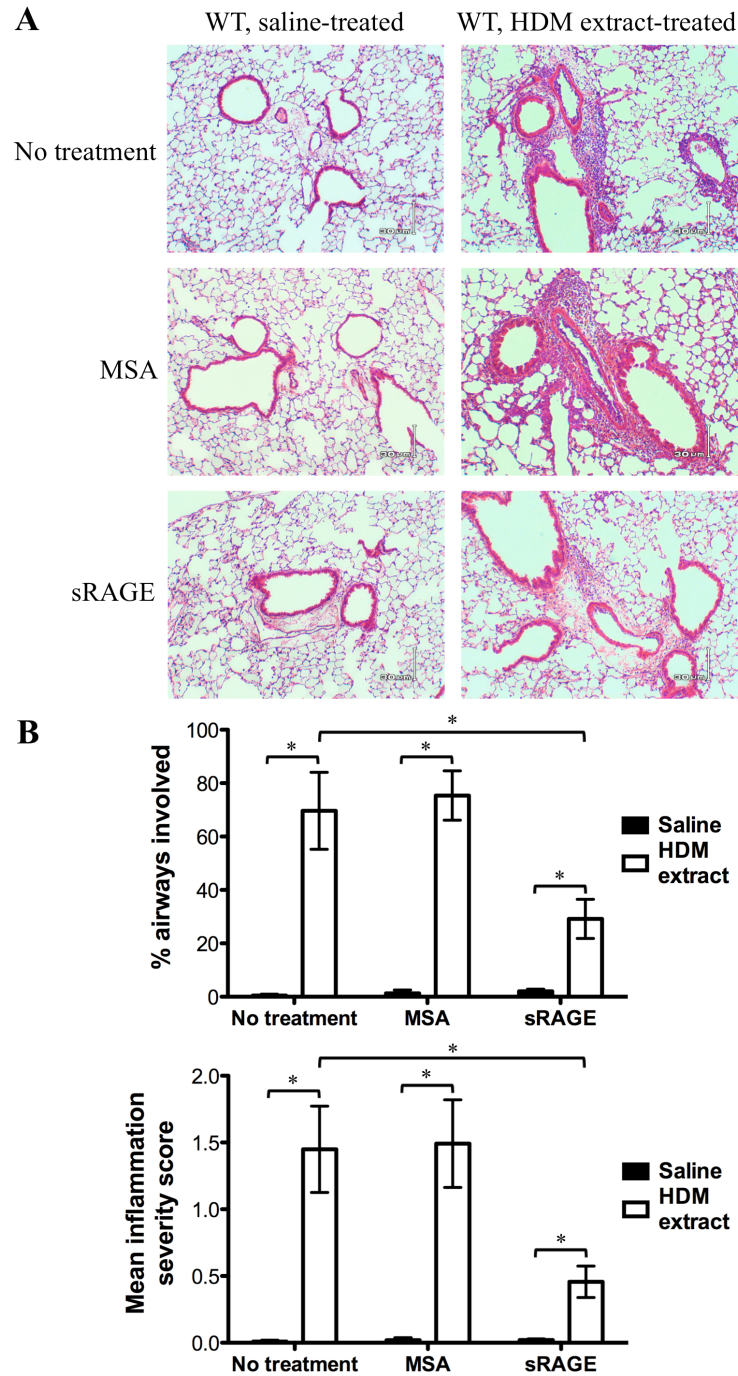


Figure 27. Exogenous sRAGE markedly attenuates peribronchial and perivascular eosinophilic infiltrates in response to HDM allergen.

(A) Representative photomicrographs recorded at 20X magnification and (B) graphical summaries of percentage airways involved and mean inflammation severity scores across all bronchovascular bundles (0 = no inflammatory infiltrate, 1 = mild inflammatory infiltrate, 2 = moderate inflammatory infiltrate, 3=severe inflammatory infiltrate), of H&E stained lung sections from mice treated with HDM extract or saline control, alongside sRAGE, MSA, or no additional protein. n=3-4 per treatment group.

4.16 BONE MARROW CHIMERIC MICE LACKING RAGE DEVELOP ALLERGIC AIRWAY DISEASE IN RESPONSE TO HDM ALLERGEN

One of the most important questions arising from studies of RAGE's role in allergic airway disease/asthma is the relative importance of pulmonary RAGE versus hematopoietic RAGE. Although localization studies discussed above suggested that eosinophils and helper T lymphocytes do not express any RAGE, and that macrophages express the protein at low levels, the literature is replete with studies suggesting expression of RAGE by inflammatory cells, and particularly by monocytes-macrophages. Thus, the possibility that macrophage RAGE rather than pulmonary parenchymal RAGE is responsible for the effects seen in models of allergic airway disease/asthma cannot be easily dismissed. To elucidate this mechanistic question, bone marrow chimeric mice were generated, radioresistant alveolar macrophages were ablated with liposomal clodronate, and the chimeric mice were subjected to an HDM model of allergic airway disease/asthma (Figure 2A). Contrary to expectation, in response to HDM extract sensitization and challenge RAGE KO mice that had received RAGE KO donor bone marrow developed the same pulmonary eosinophilia (Figure 28) and peribronchial, perivascular, and interstitial eosinophilic inflammation (Figure 29) as wild type mice. Furthermore, no distinction could be made between wild type recipient mice with RAGE KO donor bone marrow and RAGE KO recipient mice with wild type donor bone marrow: all groups demonstrated exuberant pulmonary eosinophilic inflammation in response to HDM allergen and normal quiescent lung when treated with saline vehicle control. Unsurprisingly, neither could any distinction be made between the four HDM extract-treated groups with regard to pulmonary function parameters (results not shown).

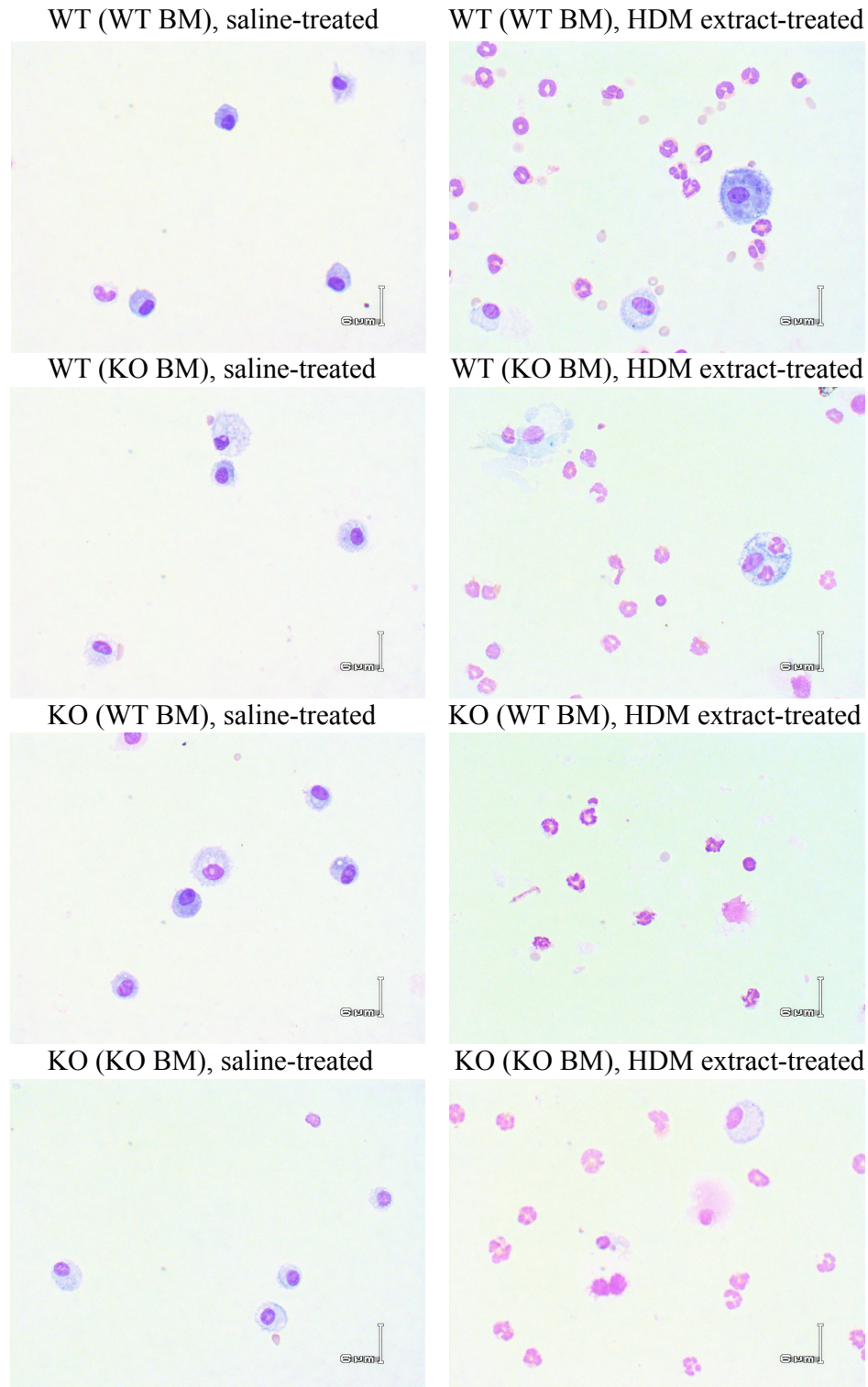


Figure 28. Bone marrow chimeric mice develop airway eosinophilia in response to HDM allergen, regardless of pulmonary epithelial or hematopoietic RAGE expression status.

Representative photomicrographs recorded at 40X magnification of Romanowsky-stained cytopspins prepared from BALF of chimeric mice treated with HDM extract or saline control. n=3-7 per treatment group.

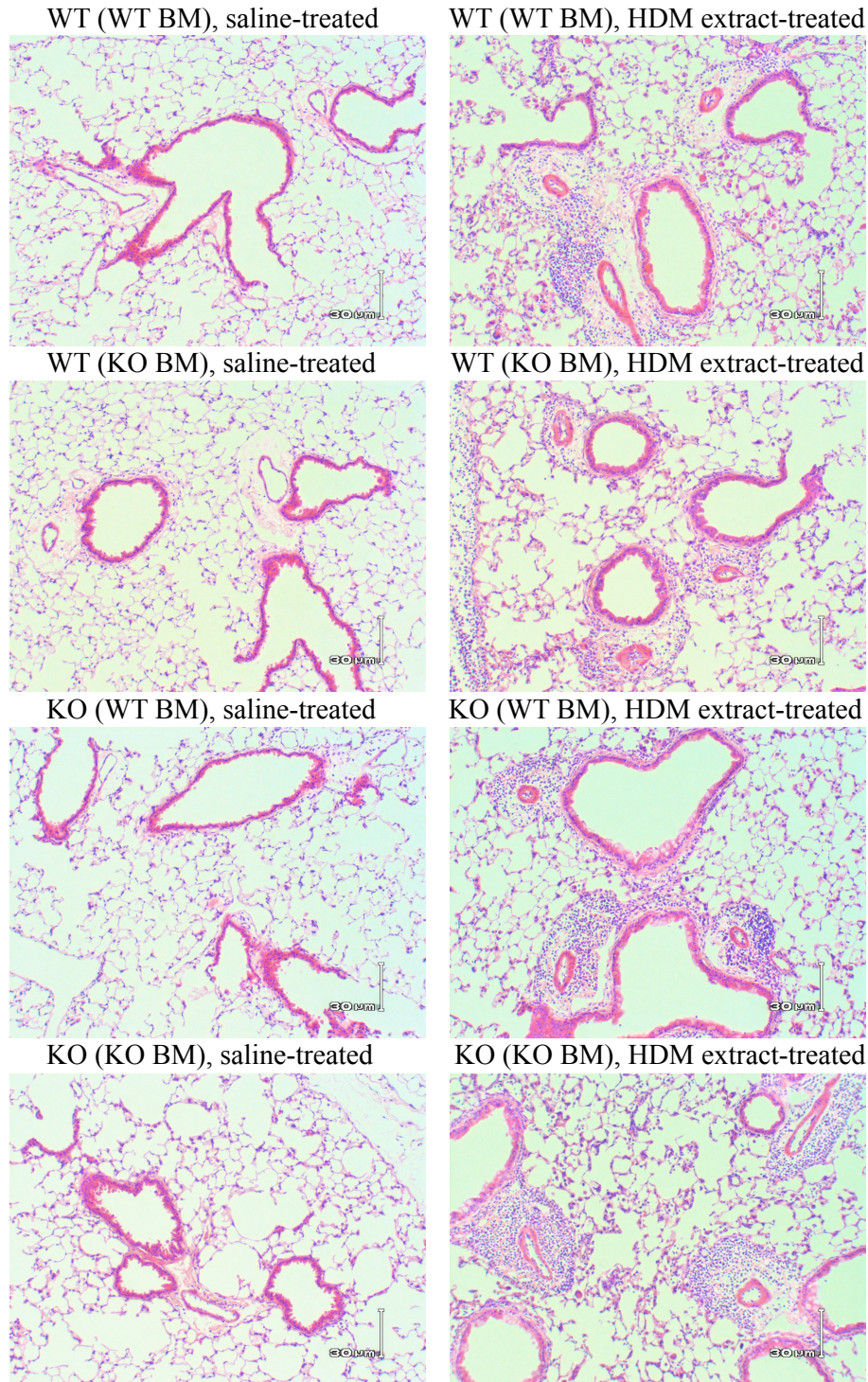


Figure 29. Bone marrow chimeric mice develop peribronchial, perivascular, and interstitial eosinophilia in response to HDM allergen, regardless of pulmonary epithelial or hematopoietic RAGE expression status.

Representative photomicrographs recorded at 20X magnification of H&E-stained lung sections from chimeric mice treated with HDM extract or saline control. n=3-7 per treatment group.

4.17 IN THE ABSENCE OF RAGE, HDM ALLERGEN DOES NOT ELICIT UP-REGULATION OF VCAM-1 EXPRESSION

To determine whether the expression levels and localization of VCAM-1 are normal in RAGE KO mice at baseline, and to determine how expression and localization are regulated in response to HDM allergen, lung sections from mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A) were inspected by immunofluorescence microscopy for the expression of VCAM-1. VCAM-1 expression was indistinguishable between wild type and RAGE KO mice at baseline, and in both cases expression was confined to the vascular endothelium. In response to HDM extract sensitization/challenge, whereas wild type mice demonstrated up-regulated VCAM-1 expression (notably at bronchovascular bundles with marked eosinophilic infiltrates), RAGE KO mice demonstrated no difference from their saline-treated control counterparts (Figure 30).

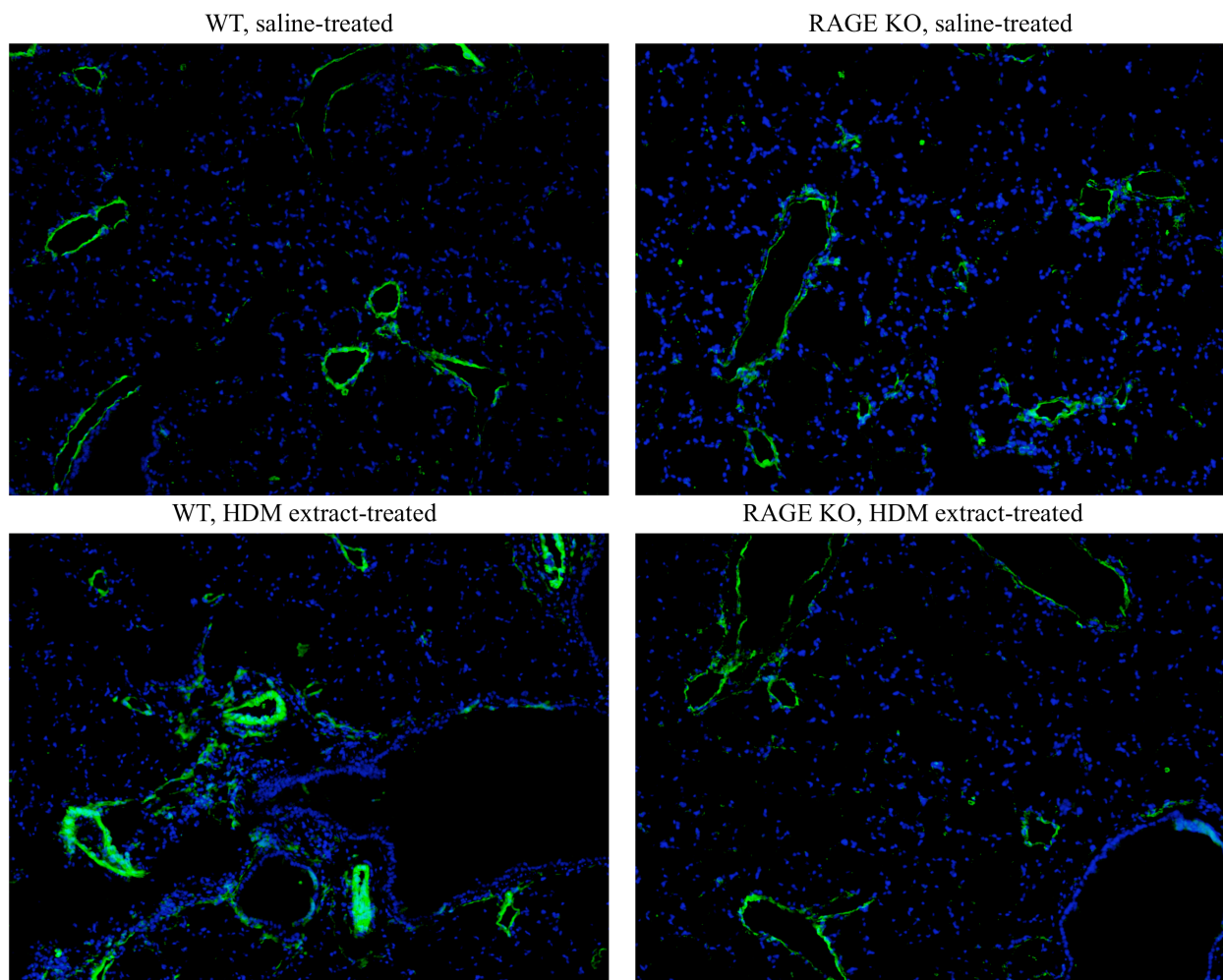


Figure 30. Mice lacking RAGE do not up-regulate vascular VCAM-1 expression in response to HDM allergen.

Representative immunofluorescence photomicrographs recorded at 10X magnification of sections of lung from mice treated with HDM extract or saline control. *Green* – VCAM-1; *blue* – nuclei.

4.18 IN THE ABSENCE OF RAGE, THE HUMORAL IMMUNE RESPONSE TO HDM ALLERGEN IS NORMAL

To determine whether the humoral immune response to HDM allergen is altered in RAGE KO mice, ELISA was performed on sera and lung homogenates of wild type and RAGE KO mice

subjected to an HDM model of allergic airway disease/asthma (Figure 2A) to evaluate the levels of HDM-specific immunoglobulin. Interestingly, significantly elevated levels of HDM-specific IgG₁ in serum (Figure 31A) and lung homogenate (Figure 31B) of both wild type and RAGE KO mice that had been sensitized and challenged with HDM extract were observed. To test whether there were broad differences between the HDM antigen binding profiles of serum IgG from allergen-primed and -challenged wild type versus RAGE KO mice, HDM extracts separated by SDS-PAGE under either reducing or non-reducing conditions and transferred to membranes were probed with sera from sensitized and challenged wild type and RAGE KO mice (Figure 31C), indicating no demonstrable difference in antigen binding profiles between the two strains and suggesting that RAGE is not directly involved in antigen recognition.

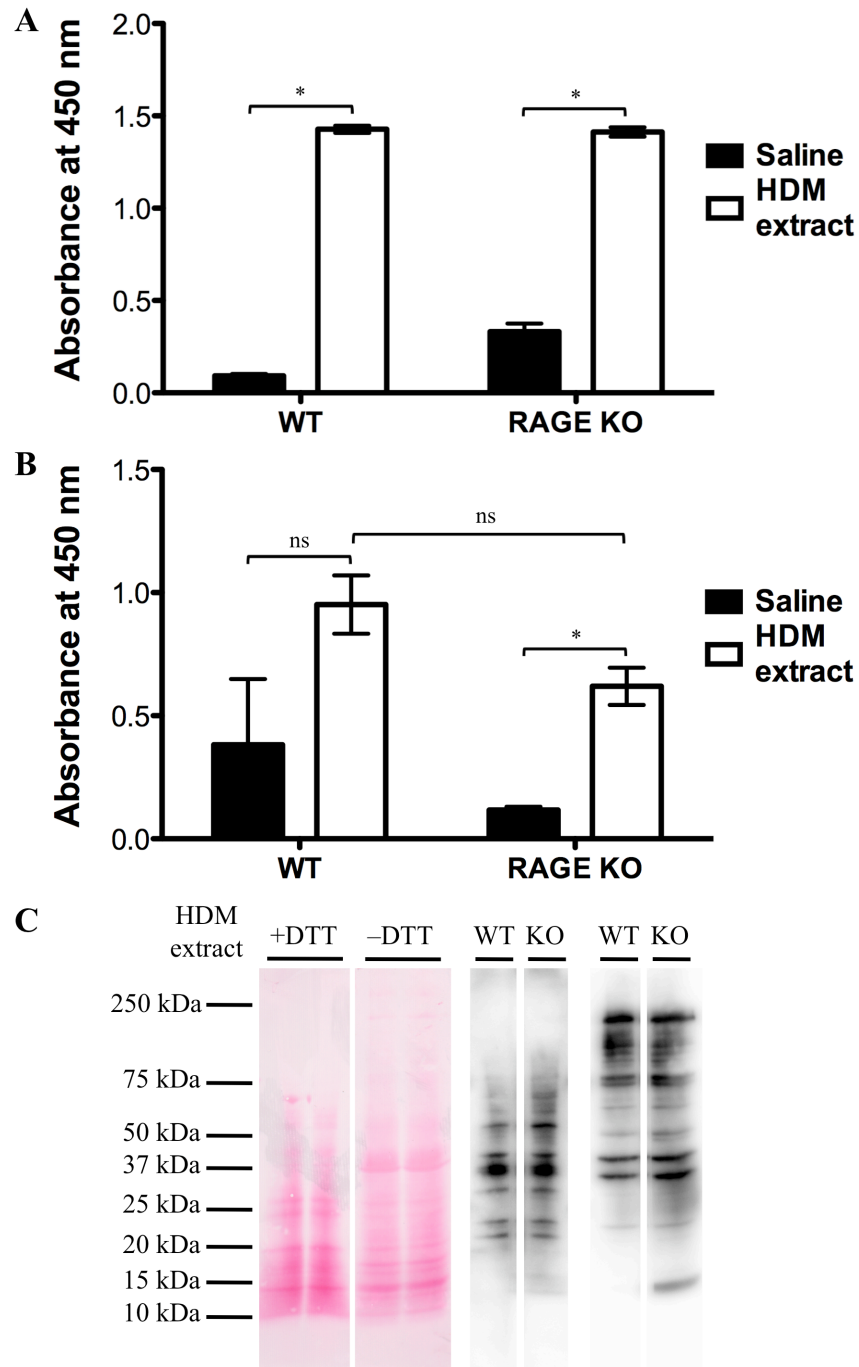


Figure 31. Mice lacking RAGE develop competent IgG antibody responses against HDM antigens indistinguishable from those of wild type mice.

ELISA shows levels of HDM antigen-specific IgG₁ antibodies in (A) sera and (B) lung homogenates from mice treated with HDM extract or saline control. (C) Left, SDS-PAGE separation under reducing and non-reducing conditions, membrane transfer, and Ponceau S stain (serves as loading control) of HDM extracts used to detect HDM-specific IgG. Right, immunoblot demonstrating HDM antigen binding by serum IgG from wild type or RAGE KO mice treated with HDM extract. n=3-10 per strain/treatment group.

To determine whether IgE production is intact in RAGE KO mice, ELISA was performed on sera of wild type and RAGE KO mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A) to evaluate the levels of total and HDM-specific IgE. Remarkably, the levels of both HDM-specific and total IgE (Figure 32) were significantly elevated in the sera of both wild type and RAGE KO mice sensitized and challenged with HDM extract, demonstrating that isotype class switching is unaffected by the absence of RAGE.

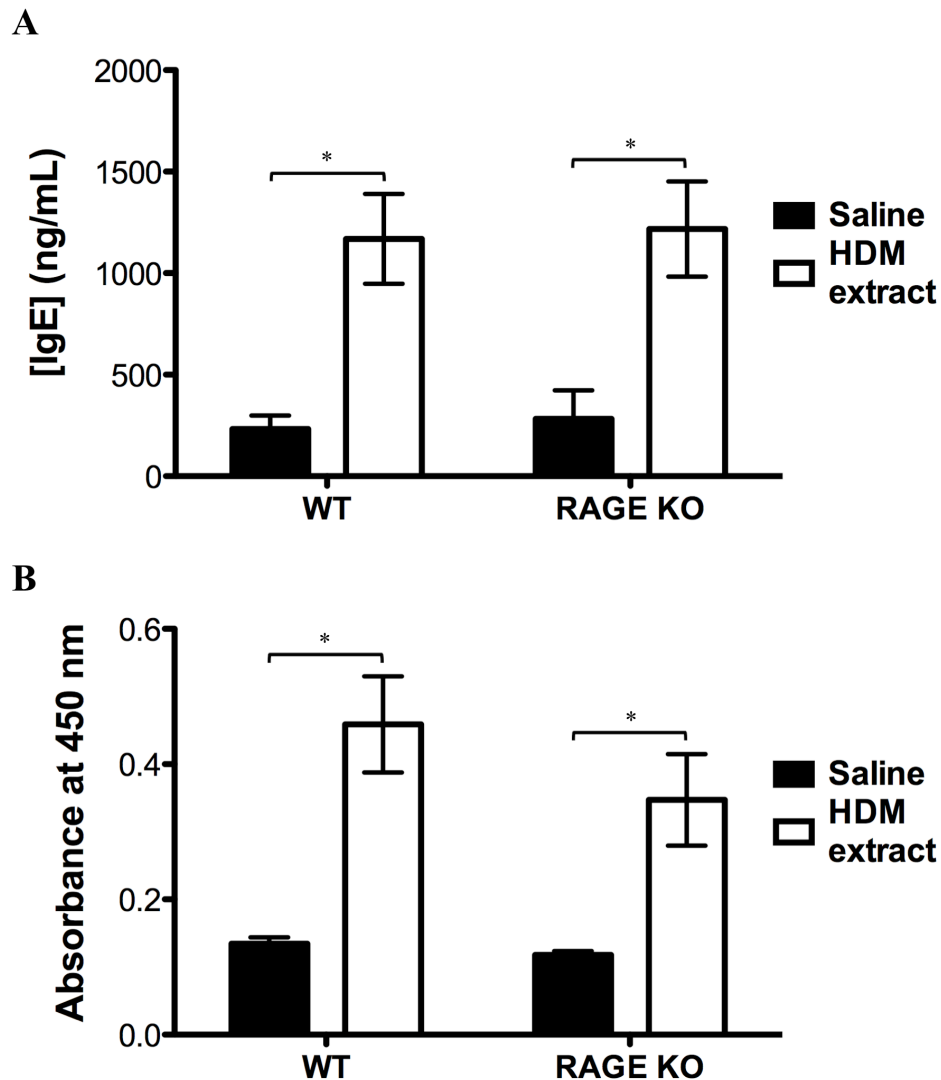


Figure 32. Mice lacking RAGE develop total and HDM antigen-specific IgE antibody responses to HDM sensitization and challenge indistinguishable from those of wild type mice.

ELISA shows levels of (A) total and (B) HDM antigen-specific IgE antibody in sera from mice treated with HDM extract or saline control. n=3-10 per strain/treatment group.

4.19 IN THE ABSENCE OF RAGE, IL-17 DEMONSTRATES COMPLEX REGULATION

Despite the lack of prominent neutrophilia in response to HDM allergen – in contrast to the florid neutrophilia in response to fungal (*Aspergillus*)^{304,305} or cockroach fecal^{306,307} allergen – analysis of IL-17 regulation in an HDM model of allergic airway disease/asthma (Figure 2A) was pursued in the interest of completeness. Because IL-17 is undetectable in BALF (results not shown), lung homogenates were tested for IL-17 levels by ELISA (Figure 33). Interestingly, IL-17 appears to have significantly heightened baseline expression in RAGE KO mice as compared to wild type mice. However, while the wild type mice demonstrate induction of IL-17 expression in response to HDM extract sensitization and challenge, the RAGE KO mice do not, maintaining IL-17 at baseline levels.

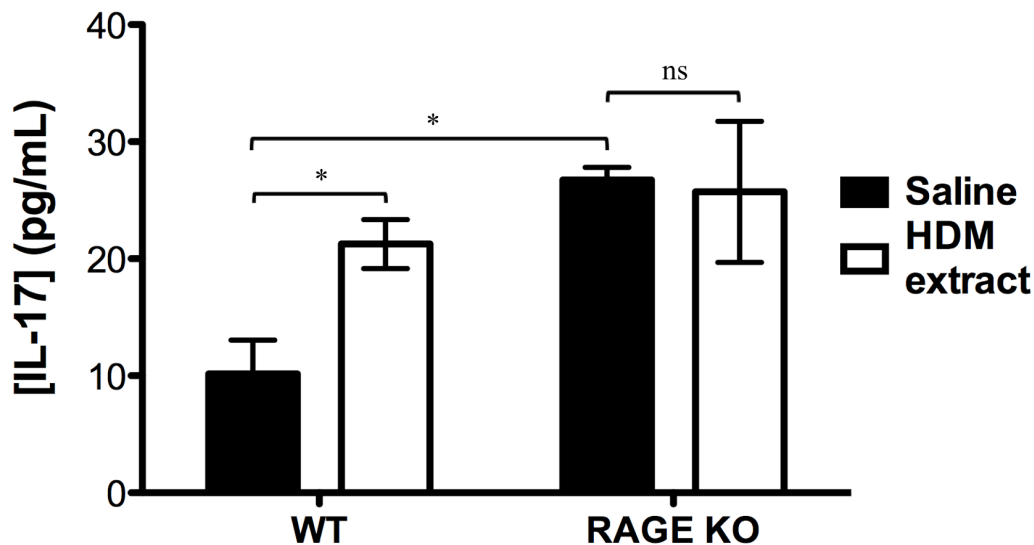


Figure 33. Mice lacking RAGE demonstrate elevated IL-17 at baseline, but do not up-regulate IL-17 expression in response to HDM allergen.

ELISA shows levels of IL-17 in lung homogenates from mice treated with HDM extract or saline control. n=3-6 per strain/treatment group.

4.20 IN THE ABSENCE OF RAGE, IL-4 EXPRESSION IN RESPONSE TO HDM ALLERGEN IS NORMAL

IL-4 is one of the classic triad of Th2 cytokines thought to play a pivotal role in allergic airway disease/asthma. To characterize the expression pattern of this cytokine in the absence of RAGE, ELISA was performed on BALF from wild type and RAGE KO mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A). As anticipated and in concordance with the antibody data, IL-4 expression is up-regulated from baseline in response to sensitization and challenge with HDM extract in both wild type and RAGE KO mice, with no significant difference between the two strains (Figure 34).

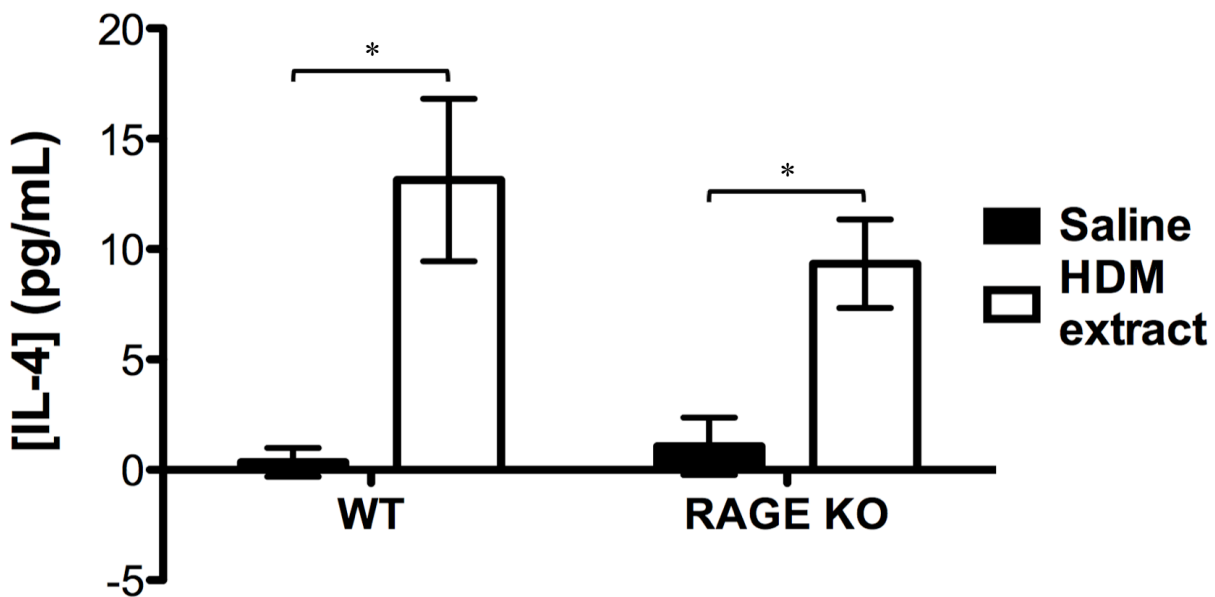


Figure 34. Mice lacking RAGE secrete IL-4 in response to HDM allergen at levels indistinguishable from those of wild type mice.

ELISA shows levels of IL-4 in BALF from mice treated with HDM extract or saline control. n=3-6 per strain/treatment group.

4.21 IN THE ABSENCE OF RAGE, EXPRESSION OF EOSINOPHIL CHEMOKINES IN RESPONSE TO HDM ALLERGEN IS ABROGATED

Because of the notable defect in eosinophil recruitment to the lung in HDM extract-treated RAGE KO mice, ELISA was performed on BALF from wild type and RAGE KO mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A) to assess the levels of eotaxins. HDM allergen-induced expression of both eotaxin and especially eotaxin-2 was severely diminished in RAGE KO mice as compared to wild type counterparts (Figure 35). This indicates that the effect of RAGE's absence on allergic airway disease is likely not direct and local (e.g. RAGE directly binding to eosinophils and mediating their ingress from the circulation into the tissue), but is rather mediated by defects in canonical eosinophil chemokine networks.

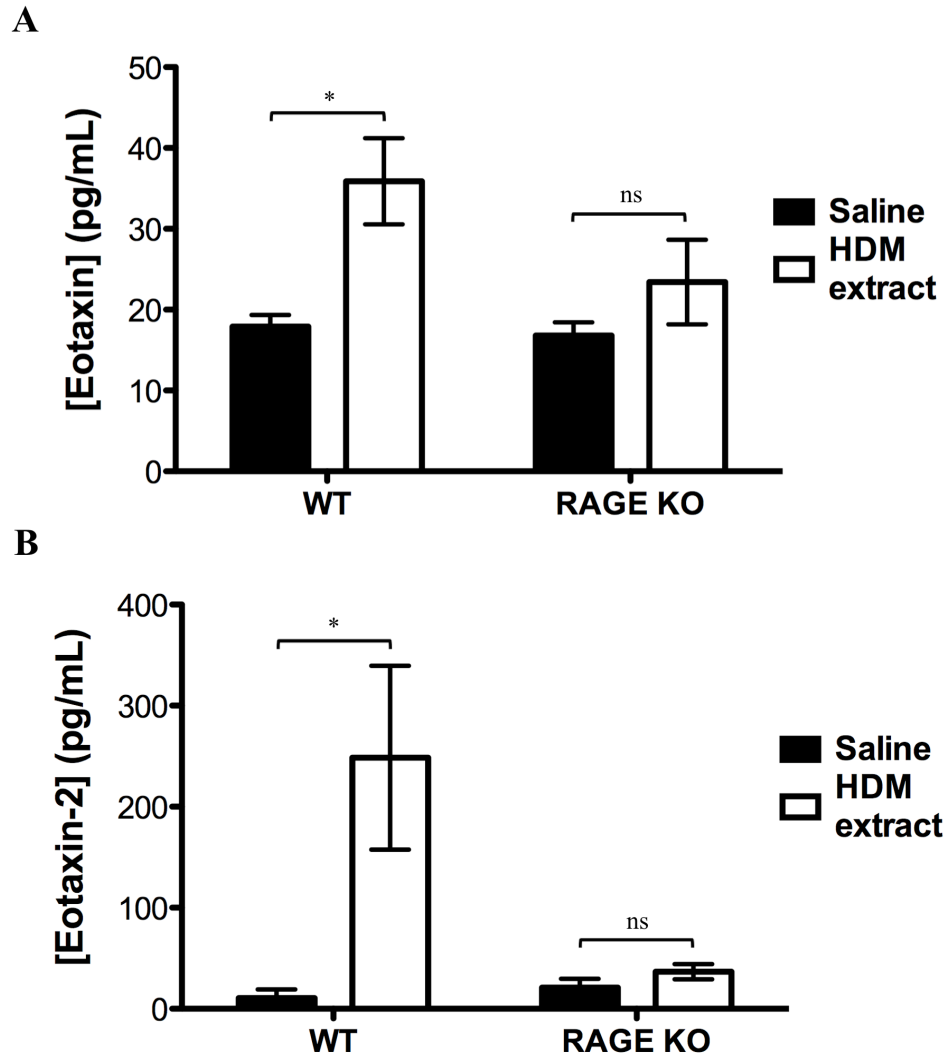


Figure 35. Mice lacking RAGE demonstrate abolished eotaxin and eotaxin-2 expression in response to HDM allergen.

ELISA shows levels of (A) eotaxin and (B) eotaxin-2 in BALF from mice treated with HDM extract or saline control. n=3-6 per strain/treatment group.

4.22 IN THE ABSENCE OF RAGE, IL-5 EXPRESSION IN RESPONSE TO HDM ALLERGEN IS ABROGATED

To characterize the expression pattern of IL-5 in RAGE's absence, ELISA was performed on BALF from wild type and RAGE KO mice subjected to an HDM model of allergic airway

disease/asthma (Figure 2A). Severe impairment in HDM allergen-driven IL-5 protein expression in RAGE KO mice, as compared to wild type counterparts, was observed (Figure 36A). Due to the centrality of IL-5 to the eosinophilic component of asthma, and in order to test whether the defect was secretory or synthetic, qRT-PCR was performed on lung homogenate transcript from the same group of mice; the IL-5 defect in RAGE KO mice occurs at the level of mRNA synthesis (Figure 36B).

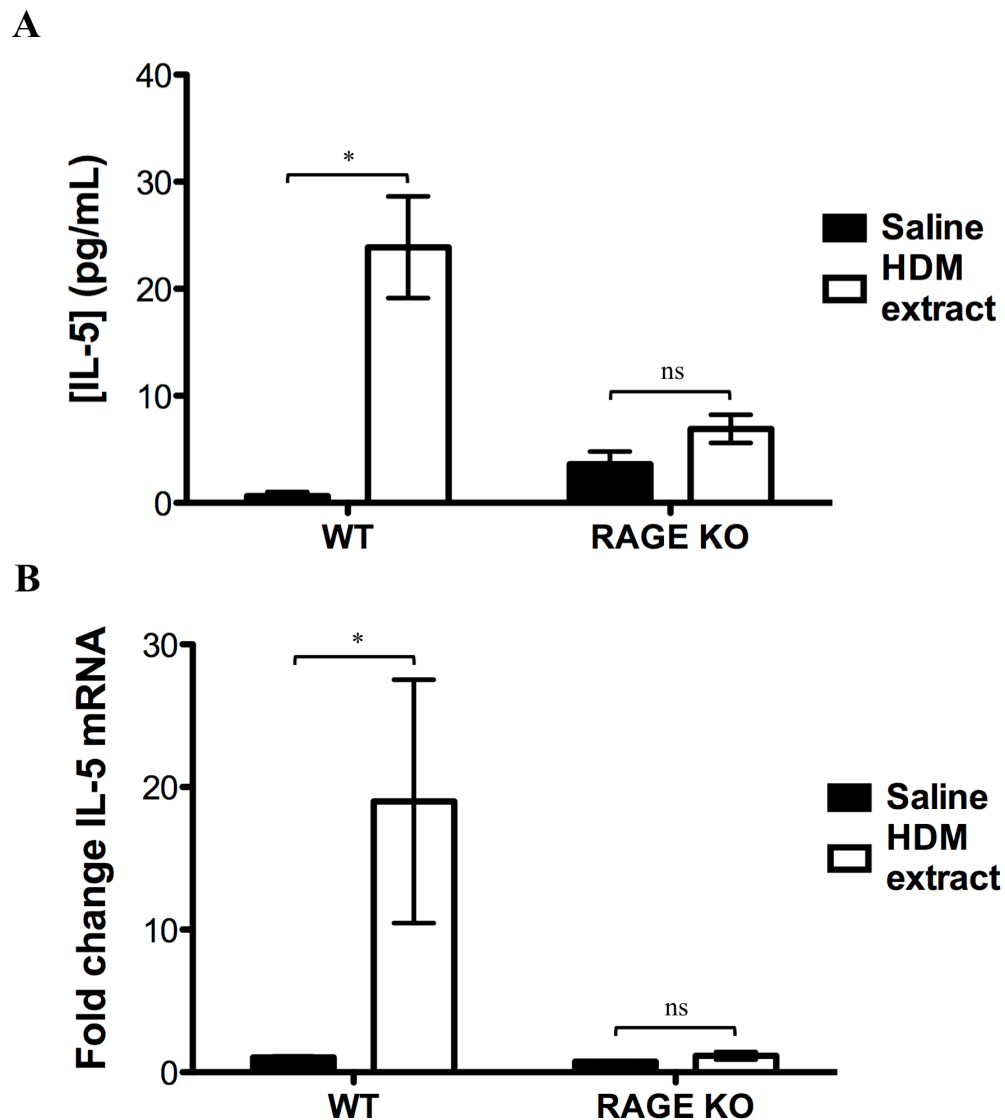


Figure 36. Mice lacking RAGE demonstrate abolished IL-5 expression in response to HDM allergen. (A) ELISA on BALF and (B) qRT-PCR (GAPDH used as housekeeping control) on lung homogenate mRNA shows levels of IL-5 in mice treated with HDM extract or saline control. n=3-6 per strain/treatment group.

4.23 IN THE ABSENCE OF RAGE, IL-13 EXPRESSION IN RESPONSE TO HDM ALLERGEN IS ABROGATED

To characterize the expression of IL-13 in the absence of RAGE, ELISA was performed on BALF from wild type and RAGE KO mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A). To test whether the effects seen occur at the level of protein secretion or mRNA synthesis, qRT-PCR was performed on lung homogenate transcript from the same group of mice. IL-13 induced in response to HDM allergen is abrogated in RAGE KO mice at both the transcript and protein levels (Figure 37).

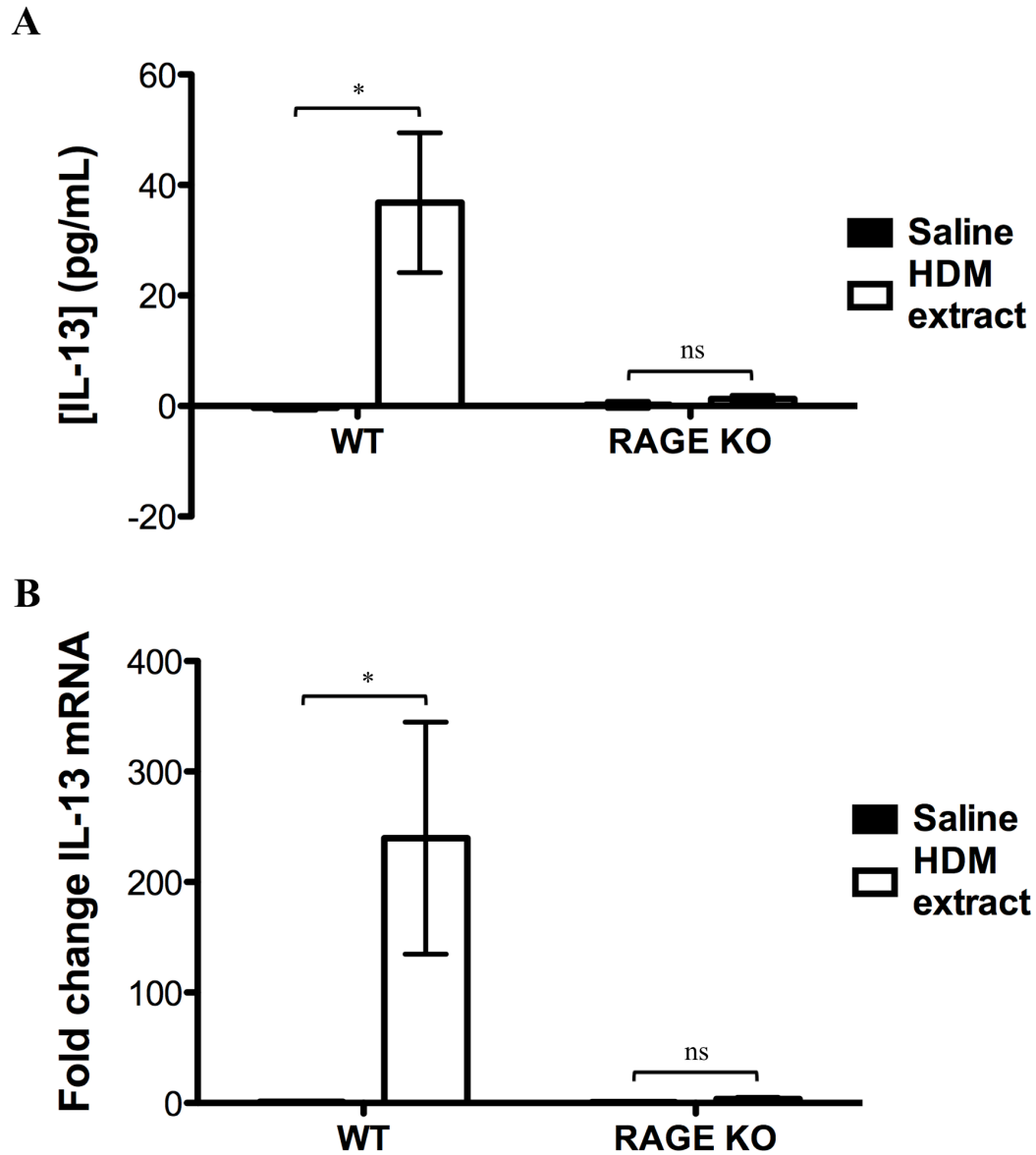


Figure 37. Mice lacking RAGE demonstrate abolished IL-13 expression in response to HDM allergen.

(A) ELISA on BALF and (B) qRT-PCR (GAPDH used as housekeeping control) on lung homogenate mRNA shows levels of IL-13 in mice treated with HDM extract or saline control. n=3-6 per strain/treatment group.

4.24 EXPRESSION OF TSLP IS UNALTERED IN RESPONSE TO HDM ALLERGEN

To understand the apparently discoordinate Th2 response in RAGE's absence, in which IL-4 and IgE secretion are intact but IL-5, IL-13, and downstream chemokines are abrogated, study of stroma-secreted cytokines was undertaken. As TSLP could not be detected in BALF, lung homogenate protein and transcript from wild type and RAGE KO mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A) were analyzed for levels of TSLP by ELISA and qRT-PCR, respectively. Both assays demonstrate no up-regulation of TSLP in either wild type or RAGE KO mice in response to HDM allergen (Figure 38).

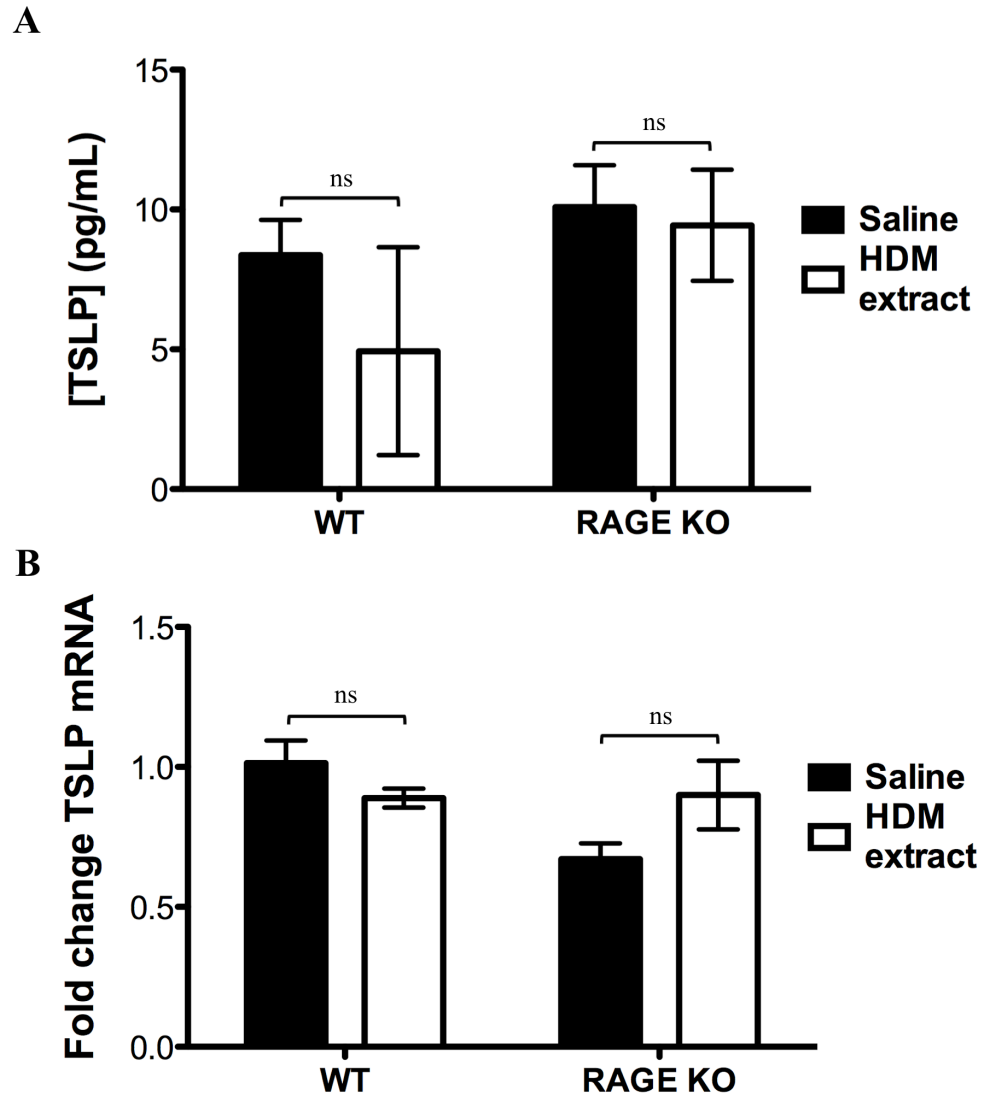


Figure 38. Both wild type mice and mice lacking RAGE demonstrate no increase in TSLP expression in response to HDM allergen.

(A) ELISA on protein and (B) qRT-PCR (GAPDH used as housekeeping control) on transcript isolated from lung homogenate shows levels of TSLP in mice treated with HDM extract or saline control. n=3-6 per strain/treatment group.

4.25 IN THE ABSENCE OF RAGE, IL-25 EXPRESSION IN RESPONSE TO HDM ALLERGEN IS NORMAL

To assay the levels of IL-25 in the absence of RAGE, ELISA was performed on BALF from wild type and RAGE KO mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A). IL-25 was up-regulated in BALF of both wild type and RAGE KO mice in response to HDM allergen, and no distinction between the two strains could be made in this regard (Figure 39).

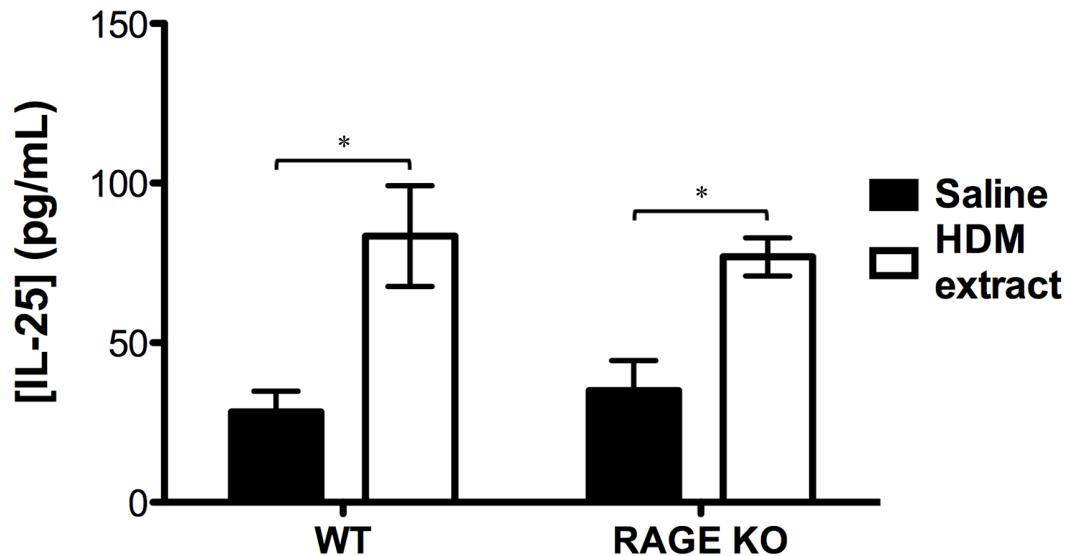


Figure 39. Mice lacking RAGE secrete IL-25 in response to HDM allergen at levels indistinguishable from those of wild type mice.

ELISA shows levels of IL-25 (IL-17E) in BALF from mice treated with HDM extract or saline control. n=3-4 per strain/treatment group.

4.26 IL-33 IS UNDETECTABLE IN BALF FOLLOWING TREATMENT WITH HDM EXTRACT

Because IL-33 is constitutively expressed in lung tissue, BALF from mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A) was assayed by ELISA for IL-33 levels. No IL-33 was detectable in BALF (results not shown). Because of the possibility that released IL-33 is very short-lived in the lung compartment, and in parallel with studies by others showing that *Alternaria* extract induced IL-33 release into BALF within 1 hour of treatment followed by rapid decay within 6 hours,³⁰⁸ IL-33 release studies were attempted. Increasing doses of HDM extract were administered intratracheally in a single dose to wild type mice and BALF was harvested at several time points thereafter. ELISA and immunoblot assays demonstrated no release of IL-33 into BALF in response to any of the doses of HDM extract at any of the time points examined (Figure 40).

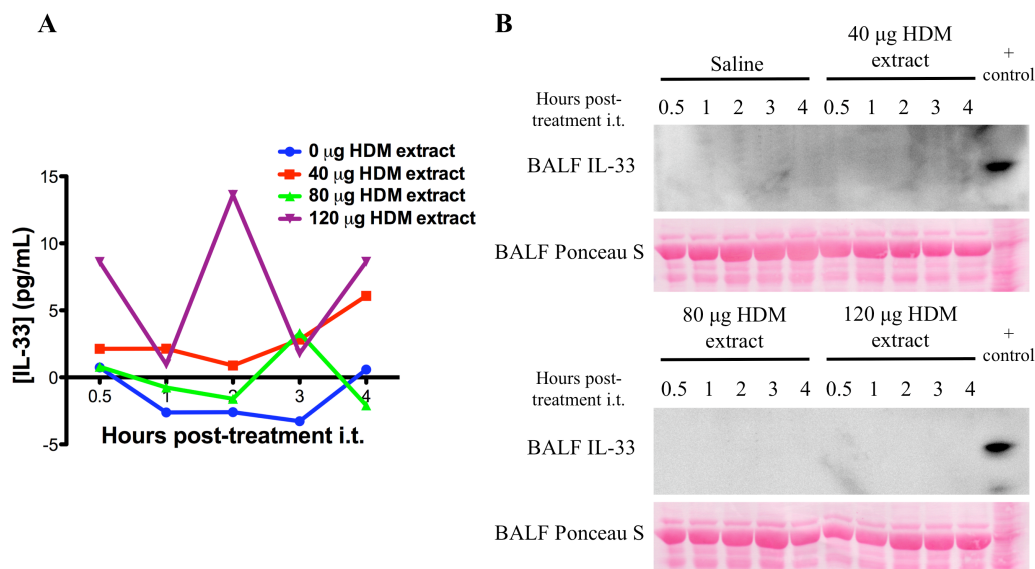


Figure 40. IL-33 release in response to increasing doses of HDM extract is undetectable.

(A) ELISA and (B) immunoblot fail to detect any release of IL-33 into the BALF in response to increasing doses of HDM extract administered i.t., at various time points after treatment. Ponceau S stain serves as loading control, and lung homogenate serves as IL-33 positive control. n=1 per dose/time point group.

4.27 IN THE ABSENCE OF RAGE, HDM ALLERGEN-INDUCED EXPRESSION OF IL-33 IS ABROGATED

As it was impossible to detect (by BALF ELISA) the release of constitutively-expressed IL-33 into airspace lining fluid in response to HDM allergen applied in a single dose i.t., or administered chronically i.n., total pulmonary IL-33 was assessed instead. Lung homogenate proteins from wild type or RAGE KO mice subjected to an HDM model allergic airway disease/asthma (Figure 2A) were analyzed by immunoblot to detect IL-33. IL-33 was markedly increased in wild type mice in response to HDM allergen, while no increase in IL-33 from baseline was observed (Figure 41A); on the contrary, a small but statistically-significant decrease in total IL-33 was observed in RAGE KO mice treated with HDM extract. Interestingly, RAGE KO mice had a somewhat elevated expression of IL-33 at baseline (compared to wild type mice), although this difference was not statistically significant. To determine whether IL-33 protein expression in lung was mirrored by transcript expression, qRT-PCR was performed on lung homogenate mRNA from the same group of mice. IL-33 transcript expression parallels (Figure 41B) total IL-33 protein expression, suggesting that differences between strain/treatment groups with respect to protein were due to differences in synthesis, rather than differences in extent of secretion (e.g. HDM extract promoting retention of IL-33 within lung cells of wild type mice but driving secretion of IL-33 from these cells into the circulation in RAGE KO mice).

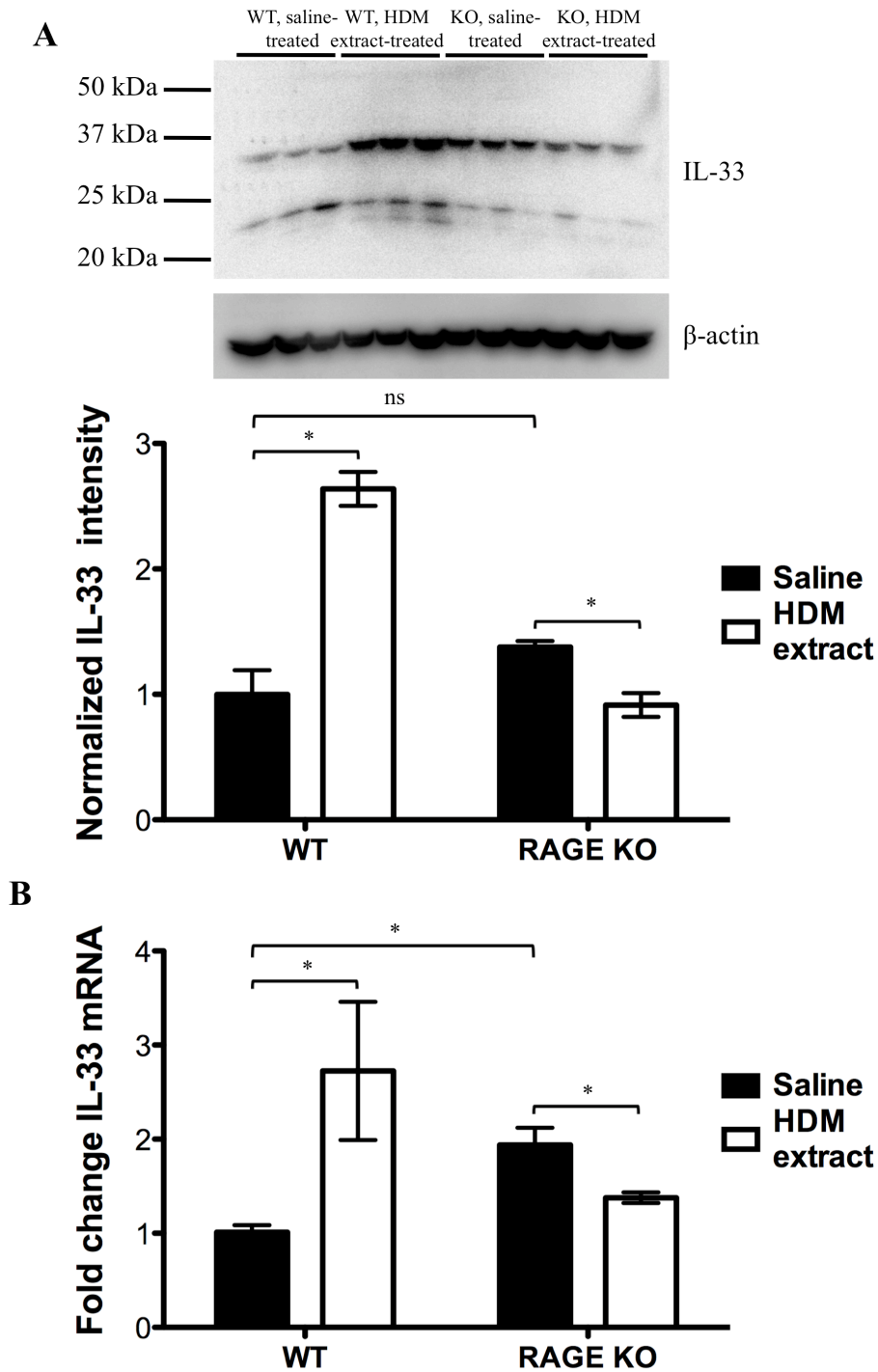


Figure 41. Mice lacking RAGE do not up-regulate IL-33 protein or transcript expression in lung in response to HDM allergen.

(A) Immunoblot (β -actin serves as loading control) and (B) qRT-PCR (GAPDH used as housekeeping control) probing for IL-33, in whole lung homogenates from mice treated intranasally with HDM extract or saline control. Densitometry analysis shows normalized IL-33: β -actin ratios for each strain/treatment group. $n=3-6$ per strain/treatment group.

4.28 IL-33 IS LOCALIZED TO SMOOTH MUSCLE AND TYPE II ALVEOLAR EPITHELIAL CELLS

As IL-33 is a potential causal antecedent to IL-5 and IL-13 expression, which is disrupted in the absence of RAGE, defining the cellular sources of pulmonary IL-33 is a first step towards establishing a structural-functional link to epithelial RAGE. To identify the sources of IL-33 in the pulmonary compartment, lung sections from mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A) were inspected by immunofluorescence microscopy for the expression of IL-33. Bronchial epithelium, type I alveolar epithelium, and most endothelium (Figure 42A-C) did not express IL-33. Some endothelial expression of IL-33 was apparent in what appear to be venules or lymphatic vessels, but no IL-33 could be detected in arterioles or alveolar capillaries.

Contrastingly, smooth muscle cells and type II alveolar epithelial cells clearly express high levels of IL-33. Peculiarly, smooth muscle cell IL-33 is most prominent in peribronchial smooth muscle, but also in vascular smooth muscle, and appears to be primarily cytoplasmic in localization (Figure 43A). Type II alveolar epithelial cell IL-33 – the bulk of pulmonary IL-33, it seems – is primarily nuclear in localization, consistent with previous reports (Figure 43B).

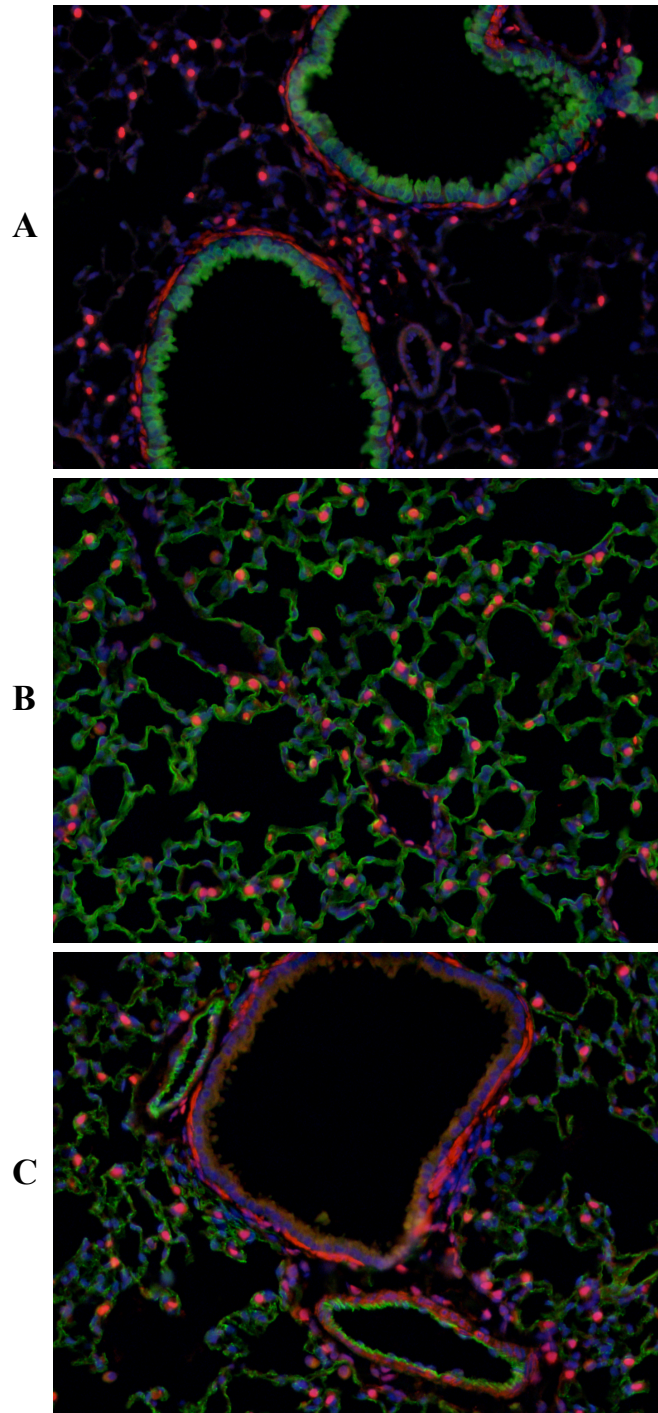


Figure 42. IL-33 is not expressed in bronchial epithelium, type I alveolar epithelium, or most endothelium.

Representative immunofluorescence photomicrographs recorded at 20X magnification of pulmonary (A) bronchial epithelial cells, (B) type I alveolar epithelial cells, and (C) endothelial cells. *Green* – CCSP/uteroglobin (bronchial epithelium), AQP5 (type I alveolar epithelium), PECAM-1/CD31 (endothelium); *red* – IL-33; *blue* – nuclei; *pink* – nuclear co-localization.

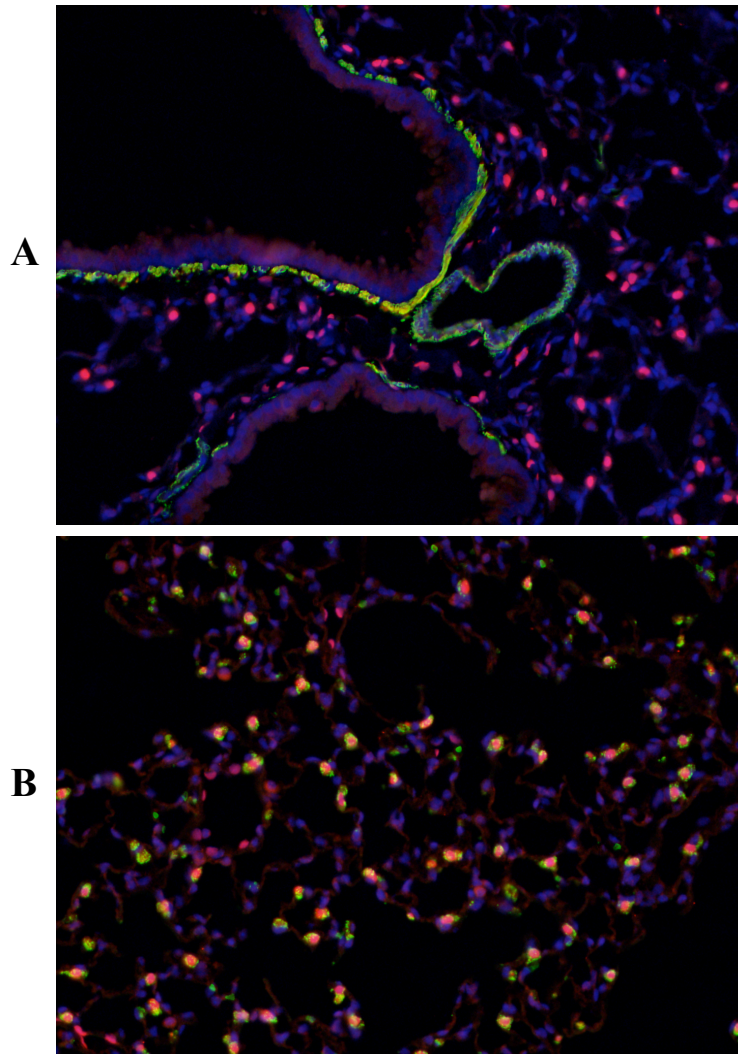


Figure 43. IL-33 is expressed in smooth muscle and type II alveolar epithelial cells.

Representative immunofluorescence photomicrographs recorded at 20X magnification of pulmonary (A) smooth muscle cells and (B) type II alveolar epithelial cells. *Green* – α -SMA (smooth muscle), proSP-C (type II alveolar epithelial cells); *red* – IL-33; *blue* – nuclei; *yellow* – cellular co-localization; *pink* – nuclear co-localization.

4.29 HDM ALLERGEN-INDUCED EXPRESSION OF IL-33 IS ATTRIBUTABLE TO INCREASED EXPRESSION OF IL-33 ON A PER CELL BASIS

The marked increase in pulmonary IL-33 expression in mice treated with HDM extract as compared to saline-treated control counterparts (and the notable difference in IL-33 expression at

baseline between wild type and RAGE KO mice) can conceivably arise from one of two mechanisms. In the first scenario, IL-33 expression is increased in response to HDM allergen due to increased numbers of IL-33⁺ cells, whether due to increased proliferation of IL-33-expressing cell types (e.g. type II alveolar epithelial cells) or due to HDM-mediated recruitment of novel IL-33-expressing cells (e.g. a larger fraction of smooth muscle cells expresses IL-33, or additional cell types are induced to express IL-33). In the second scenario, IL-33 expression is not up-regulated in response to HDM allergen by recruiting new cells to express IL-33; it is rather the IL-33 expression on a per cell basis that is altered. To address this question, immunofluorescence microscopy was performed on lung sections from wild type and RAGE KO mice subjected to an HDM model of allergic airway disease/asthma (Figure 2A). Sections were inspected for changes in IL-33 localization with reference to both treatment and strain. No difference in the spectrum of cell types expressing IL-33 was observed between wild type and RAGE KO mice, nor between HDM extract-treated and saline control-treated mice (Figure 44A). A quantitation method was implemented to determine the average density of IL-33⁺ cells per section for each strain/treatment group (Figure 44B), taking into account the cellularity of the specimens assayed by normalizing to a nuclear stain. No significant difference in the density of IL-33⁺ cells between groups was detectable, indicating that the marked up-regulation of IL-33 protein and transcript in response to HDM allergen in wild type mice, as well as the difference in baseline expression of IL-33 between wild type and RAGE KO mice, was attributable to an increase in IL-33 expression on a per cell basis. Indeed, this is easily seen on visual inspection, as the intensity of type II alveolar epithelial cell IL-33 signal was substantially greater in HDM-treated wild type mice than in the other strain/treatment groups (Figure 44A, left lower panel).

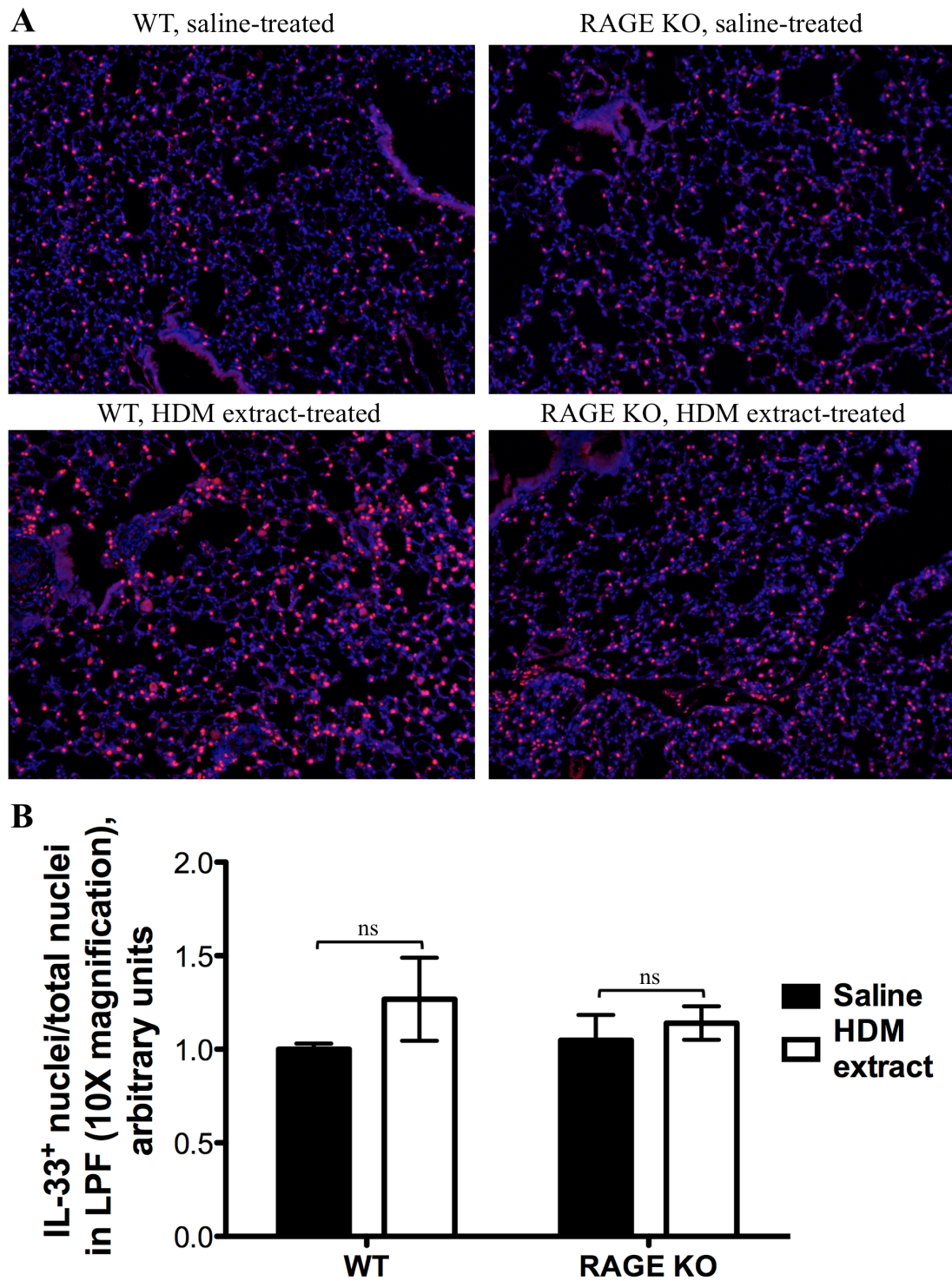


Figure 44. No difference in the distribution or density of IL-33-expressing cells is observable between strains or treatments in an HDM model of allergic airway disease/asthma.

(A) Representative immunofluorescence photomicrographs recorded at 10X magnification and (B) normalized density of IL-33⁺ cells per section, in lung sections from mice treated intranasally with HDM extract or saline control. n=3-4 per strain/treatment group. *Red* – IL-33; *blue* – nuclei; *pink* – nuclear co-localization.

5.0 DISCUSSION

5.1 SOLUBLE RAGE BIODISTRIBUTION AND CLEARANCE

Of the three common routes of administration tested – intravenous, intraperitoneal, and intratracheal, sRAGE can be delivered to the pulmonary compartment solely via intratracheal instillation. These findings cast doubt on studies suggesting a therapeutic effect of intraperitoneally-administered sRAGE in mouse models of lung disease.³⁰⁹ It is possible that in the sRAGE preparations used by other investigators there are contaminants present, such as LPS, which could ameliorate pulmonary disease by rerouting pathogenic cell populations away from the lung and to the peritoneum, or by inducing such populations in the lung to deviate to a less pernicious phenotype. Alternatively, it is also possible that the low doses (hundreds of nanograms) of radiolabeled proteins given here failed to recruit low-affinity transport receptors in the pulmonary vasculature that become relevant when much higher doses (dozens of micrograms) of sRAGE are given i.p. or i.v.

A secondary consideration to this set of experiments' primary aim of determining the efficacious route(s) of pulmonary delivery of sRAGE was that these studies also afforded the opportunity to probe for potential receptors for sRAGE. Indeed, the MSA treatment controls were incorporated with this in mind, to distinguish between specific preferential organ biodistribution due to sRAGE binding partners (where the measure of biodistribution is

radioactivity per unit mass) and nonspecific preferential organ biodistribution due to greater vascular density or more leaky vessels. At first glance, the biodistribution of sRAGE and MSA following i.p. or i.v. administration would seem to suggest preferential retention of sRAGE (as compared to MSA) in a number of organs, including kidneys, liver, spleen, stomach, small intestine, colon, pancreas, skeletal muscle, bone, and brain. However, when these two administration routes were compared to each other and to i.t. instillation, no consistent preferential organ biodistribution emerged except for the kidneys. Indeed, this phenomenon (of sRAGE apparently distributing more effectively than MSA to many organs) was likely not due to latent sRAGE binding sites present in these organs, but rather to more rapid kinetics of transport of sRAGE (roughly half the molecular weight of MSA, with a reported Stokes radius of 2.81 nm for human sRAGE³¹⁰ as compared to a Stokes radius of 3.55 nm for bovine serum albumin³¹¹) across the medium-sized (~4-5.5 nm in diameter) and largest (~25 nm in diameter) pores of the peritoneal membrane.³¹² In turn, with peritoneally-delivered sRAGE entering the circulation more rapidly than MSA, a transient discrepancy in distribution between the two radiolabeled proteins arises, which resolves as the proteins are excreted. The preferential biodistribution of sRAGE to the kidneys appears to have been a *bona fide* effect and likely relates to the size and charge properties of sRAGE and MSA. sRAGE binds with high affinity to heparin and heparan sulfate (the latter is abundant in renal basement membranes) and is sufficiently small to traverse the glomerular barrier during filtration (the effective glomerular pore size being ~8 nm) and thus be excreted intact. In contrast, albumin's negative charge and larger size preclude it from being filtered intact, and therefore this protein must be eliminated via other pathways. In retrospect, a more suitable protein control for sRAGE may have been irrelevant Fab fragment, which is closer in size to sRAGE and is physiologically more inert than serum albumin.

Exogenously-administered sRAGE is rapidly cleared from the lung with clearance kinetics similar to that of MSA. Moreover, sRAGE remains intact in the lung and effectively distributes to the alveoli, from whence it translocates into the circulation. These results are surprising, as the lung is rich with many RAGE binding partners, including heparin,³⁷ heparan sulfate,³⁶ collagen I and IV,³⁸ and RAGE itself³¹⁰ (RAGE and specifically sRAGE displays homo-oligomerization at low concentrations), all of which are also present in the alveolar basement membrane and/or pulmonary interstitium. This would suggest that despite the abundance of RAGE binding partners in the lung, in the healthy state these do not act as effective sites of sRAGE sequestration. It is important to note, however, that a number of previous studies have found an inverse relationship between molecular size and clearance rate from the lung of a variety of molecules.^{313,314} Therefore, as a significantly smaller molecule than albumin, sRAGE would be expected to demonstrate more rapid clearance. That sRAGE clearance parallels that of MSA, and is thus less rapid than predicted, may suggest retardation of sRAGE transport by transient interactions between sRAGE and its ligands. However, it is also important to note that an alveolar receptor involved in albumin uptake and transcytosis, gp60, has been identified,³¹⁵ and it is possible that such a designated system for return of protein into the circulation does not exist for sRAGE. If sRAGE clears predominantly or exclusively via slower, nonspecific mechanisms such as paracellular transport and fluid-phase endocytosis,³¹⁶ albumin may thus not serve as a suitable control to infer a subtle ligand-mediated retardation in sRAGE egress. Regardless of the microscopic details of protein clearance from the lung, it is evident from these biodistribution and clearance studies that, should sRAGE be used as a therapeutic in pulmonary disease – assuming no additional functionalization, such as PEGylation to increase half-life – it must be administered frequently (one or more times daily) and via the airways. These studies

utilized an i.t. instillation approach, but they also imply that intranasal and nebulized aerosol administration would exhibit substantial efficacy of sRAGE delivery.

5.2 EOSINOPHIL RECRUITMENT STUDIES

No specific eosinophil-recruiting capacity of exogenous lung-administered sRAGE could be detected. In these studies, care was taken to use species-matched endotoxin-depleted proteins so as to avoid both an allergic response to foreign epitopes and neutrophil chemotaxis in response to LPS. Multiple-dose experiments were incorporated to accommodate the possibility that an induction dose of sRAGE was necessary to elicit an appreciable response to a second application of sRAGE. This would simulate a scenario in which a disease process first causes epithelium to shed sRAGE, which then acts in an autocrine, paracrine, or endocrine fashion on local or distant intermediary actors or terminal effectors to up-regulate responders on which subsequently-shed sRAGE acts. No specific eosinophil ingress in response to sRAGE was seen in the multiple-dose experiments, either. Thus, it may be concluded that sRAGE does not have the capacity to induce eosinophil chemotaxis when delivered at high doses to the lung. Other investigators have suggested that exogenous sRAGE (~100 µg, twice what was delivered in the studies described herein) induces pulmonary influx of monocytes and neutrophils; while this may certainly be a genuine effect of supraphysiologic doses of sRAGE, it is also possibly a consequence of residual LPS in the preparations used. The finding that bovine sRAGE was previously shown to be effective at inducing eosinophil chemotaxis may be reflective of an allergic phenomenon, as there is substantial discrepancy in amino acid sequence between bovine and murine RAGE.

5.3 RAGE AND ASTHMA PHENOTYPE

The results of this study suggest that RAGE is a key mediator in the disease mechanisms that eventuate in airway hyperresponsiveness, mucus secretion and airway remodeling, and pulmonary eosinophilia in the HDM model of allergic airway disease/asthma. Taking into consideration the great difference in response between wild type and RAGE KO mice to HDM allergen, it is perhaps surprising that RAGE levels and localization in the pulmonary compartment are essentially unchanged between allergen- and saline control-treated groups. This suggests the possibility that it is not RAGE itself, but rather a protein whose expression is tightly linked to RAGE, that is responsible for the effects seen in RAGE KO mice. While this possibility cannot be definitively excluded, given the observation that treatment with sRAGE – a decoy for RAGE ligands – specifically and markedly reduces bronchovascular inflammation in response to HDM allergen, it would seem that the lack of RAGE itself, rather than a compensatory process occurring in genetic knockouts, is responsible for the effect seen. The lack of sRAGE ameliorating effect on pulmonary function measures is likely attributable to incomplete ablation of pulmonary eosinophilia (or, more directly, residual allergy-promoting cytokine expression). Furthermore, the sRAGE treatment studies point to the membrane protein being the pro-inflammatory isoform, consistent with much of the RAGE literature. The sRAGE treatment studies also indicate that manipulation of the RAGE signaling axis via administration of sRAGE, antagonistic antibodies, or small molecule inhibitors, may have promise in the treatment of allergic airway disease/asthma.

Significant sRAGE shedding has been noted in models of acute lung injury, and a number of studies have suggested that sRAGE is either increased or decreased in BALF of human asthmatics. sRAGE shedding may be a phenomenon unrelated to disease pathogenesis, as

the synthesis and release of parenchymal or inflammatory cell proteases during injury can account for nonspecific shedding of bystander proteins. In addition, in the absence of aggressive protease inhibition, the estimated relative abundance of the various RAGE isoforms on immunoblot is progressively shifted towards sRAGE (unpublished observations). Therefore, the observation that there is no sRAGE detected in BALF or serum of mice treated with HDM extract is not particularly concerning. It is possible that the BALF and serum preparations analyzed were too dilute and required concentrating in order to detect sRAGE, or that a low level of detectable sRAGE was cleared from the lung between the last allergen challenge and sacrifice. It is also possible that in asthma models the disease milieu is insufficiently proteolytic to induce nonspecific sRAGE shedding. Indeed, in the OVA model, challenge with allergen does not induce shedding of detectable sRAGE into BALF or serum (results not shown).

Due to its multivalency, RAGE has for some time been considered by many to be a pattern recognition receptor (PRR).^{45,317-319} To date, however, no exogenous ligand has been discovered. In our own studies addressing this question, milligram-scale quantities of HDM extract were passed over an sRAGE-activated matrix; only nanogram-scale quantities of sRAGE-bound protein were eluted, and among the identified species were none that have been characterized as important molecular mediators of allergy. Towards deciphering the relevance of allergen identity to the RAGE effects seen, the ovalbumin model of allergic airway disease/asthma was used for comparison. The findings recapitulated those of the HDM model: RAGE KO mice sensitized and challenged with ovalbumin do not develop the airway hyperresponsiveness, pulmonary eosinophilia, or goblet cell hyperplasia seen in wild type mice thus sensitized and challenged. That two very different models of allergic airway disease/asthma point to the same conclusion regarding RAGE function suggests the general applicability of the

findings and provides added incentive to explore the role of RAGE in human asthma, in which allergen triggers are diverse. Whereas in the HDM model, a physiologic allergen with intrinsic protease activity is applied intranasally to effect sensitization and challenge, in the OVA model sensitization occurs in the mesentery: the ovalbumin (which is not a particularly relevant allergen in clinical asthma) requires LPS contamination to have adequate immunogenicity, and is given with alum adjuvant intraperitoneally; challenge then occurs via the intranasal route. Given the substantial differences in the sensitization process between the HDM and OVA models, both as to location and mechanism, and the lack of structural homology between HDM proteins and ovalbumin motifs, it is reasonable to conclude that RAGE is not directly acted upon by allergen. Rather, allergen likely induces expression and release of one or more endogenous mediators that then act as activating ligands on the RAGE signaling axis.

To evaluate the plausibility of the endogenous RAGE ligand hypothesis, lung homogenates were passed over an sRAGE-activated matrix and sRAGE-bound proteins were eluted, a large proportion of which are unlikely to be of biologic significance, e.g. histones and protein translation factors, which likely associate with RAGE via large bridging molecules and nonspecific electrostatic interactions. Although broadly termed “RAGE ligands” in this discussion, such species would technically not be RAGE ligands in either the biochemical or physiologic sense. The identification of several binding partners known to be dysregulated in human asthma or murine models of the disease, such as biglycan and decorin, confers a degree of validity to the affinity chromatography approach and suggests that more stringent methods of discovering RAGE-ligand interactions, such as co-immunoprecipitation, may provide further mechanistic insight into the initiating triggers of allergic airway disease/asthma.

Immunoglobulin data lend further support to the notion of endogenous RAGE ligands acting as intermediaries between allergen and RAGE signaling cascades. The notable immunoglobulin response in serum and lung homogenate rules out the possibility that RAGE KO mice suffer from global shutdown of adaptive immunity or are somehow ignorant or anergic to antigen, and suggests that antibody homing to the site of allergenic insult – the lung – is intact. That effective immunoglobulin production in response to antigens occurs in the absence of RAGE, despite the lack of other features associated with allergic airway disease, is consistent with prior studies suggesting that B cell deficiency (and hence antibody production) does not affect the physiologic and pathologic changes seen in response to allergen in an OVA model of allergic airway disease/asthma.³²⁰ It also underscores the overall picture of RAGE – not as an antigen-sensing initiator of allergic immunity (which, if true, would be reflected in RAGE KO mice with markedly lower HDM-specific immunoglobulin levels and perhaps immunologic ignorance of one or more HDM epitopes) – but as a post-sensitization pathogenetic mediator of allergic airway disease/asthma. Additionally, the fact that in the presence of high levels of IgE the RAGE KO mice do not develop airway hypersensitivity, eosinophilia, or remodeling would seem to indicate that IgE by itself does not play a major role in the development of frank allergic disease, at least in this mouse model. However, in this regard it must be noted that changes in airway physiology were assayed using methacholine rather than allergen as the challenge agent.

Which of pulmonary or hematopoietic RAGE are critical for the effects seen in mouse models of asthma is a question that could not be clarified by these studies. Immunofluorescence microscopy indicated that two key populations in asthma, eosinophils and helper T lymphocytes, do not express RAGE. Macrophages appeared to express RAGE at low levels in naïve mice, primarily at the surface. In response to HDM treatment, macrophage RAGE appears to be

localized to the cell interior, and is notably more punctate. Whether macrophage RAGE protein expression is reflective of gene expression or phagocytic uptake of shed epithelial sRAGE is unclear. To clarify the relative importance of hematopoietic or pulmonary RAGE, bone marrow chimeric mice were generated and macrophages were depleted using liposomal clodronate prior to initiating the allergic airway disease/asthma protocol. This approach had successfully demonstrated the importance of parenchymal TLR4 in HDM models of allergic airway disease/asthma.³²¹ Contrary to expectation, RAGE KO mice that received RAGE KO bone marrow developed the same peribronchial, perivascular, and interstitial eosinophilic inflammation in response to HDM extract that wild type mice did. There are several potential explanations of this phenomenon. First, this may be a simple consequence of greater age: the bone marrow chimeric mice were considerably older than non-chimeric mice, because of waiting times between the transplantation and macrophage ablation, and between the macrophage ablation and commencement of the HDM treatment protocol. Lack of RAGE does lead to spontaneous pulmonary fibrosis in aging mice, which no doubt arises from altered gene and protein expression. Second, it may be a consequence of the irradiation itself, which is known to lead to changes in protein expression in lung.³²²⁻³²⁵ Third, it may be an off-target effect due specifically to the clodronate treatment. Fourth, it may reflect a peculiarity of the bone marrow engraftment, wherein hematopoietic stem cells transplanted into adult animals are primed to differentiate into cells that respond to allergen differently (in this case, presumably an enhanced sensitivity) than cells arising from a hematopoietic compartment that initially developed in embryonic life. At any rate, a different approach – namely, RAGE deletion specifically in either lung or bone marrow – must be used to answer important questions regarding the relative

importance of hematopoietic vs. parenchymal RAGE in the development of allergic airway disease/asthma.

5.4 RAGE AND LYMPHOCYTE-DERIVED CYTOKINES IN ASTHMA

In the absence of RAGE, the response to antigen under conditions that foster a Th2 allergic-type response is markedly discoordinate: while robust IgG and IgE responses against allergen were generated, this was not associated with pulmonary eosinophilic inflammation, mucus hypersecretion, or airway hyperresponsiveness to methacholine challenge. To understand this complex phenotype, attention was turned to the more proximal element in the chain of causality: the network of T lymphocyte-derived cytokines and downstream effectors.

IL-17 has been suggested to play a role in allergy and asthma; in the HDM model of asthma/AAD, IL-17 is up-regulated in response to allergen sensitization and challenge in wild type mice.³²⁶ That RAGE KO mice demonstrate no IL-17 induction in response to HDM extract, and furthermore show heightened baseline expression of IL-17 in the absence of antigen, suggests that RAGE plays an important role in basal IL-17 regulation. RAGE may actively inhibit the secretion of IL-17, either in a direct fashion or by disrupting precursor cytokines such as IL-23. Conversely, the absence of RAGE may lead to compensatory induction of other factors that in turn drive IL-17 expression. As discussed previously, IL-17 has a complex role in asthma in that it promotes the neutrophilic subtype while also negatively regulating established allergic asthma by shifting the immune response away from Th2-type inflammation. The baseline elevation of IL-17 in RAGE KO mice may thus impede the initiation of a primary asthmatic response; moreover, it may account for the subtle differences in airway responsiveness to

methacholine challenge between naïve saline-treated wild type and RAGE KO mice (RAGE KO mice treated with saline demonstrated an elevated Rn parameter as compared to wild type mice thus treated), as a number of studies have indicated a role for IL-17 in promoting airway hyperresponsiveness.^{240,241,327,328} As a full allergic/asthmatic response is not initiated in the RAGE KO mice treated with HDM extract, it is not particularly surprising that in this strain there is no allergen-induced up-regulation in IL-17 secretion from baseline.

Innate and adaptive immune response mechanisms that drive humoral immunity are intact in the absence of RAGE, including antigen recognition, processing, and presentation; B lymphocyte production of immunoglobulin; and isotype class switching, this latter phenomenon being driven by IL-4. Consistent with this, IL-4 was elevated in both wild type and RAGE KO mice in response to HDM allergen. While T lymphocytes are thought to be the major source of IL-4, IL-5, and IL-13, other cell types have been linked to the production of one or more members of this triad. Thus, IL-4 induction by HDM allergen could be reflective of a partial T lymphocyte response, or of the recruitment of other cell types that produce IL-4, such as macrophages³²⁹ or basophils.³³⁰

Eosinophil chemotaxis is mediated by a diverse group of chemokines, including eotaxin, eotaxin-2, RANTES, MCP-3, MCP-4, and MIP-1 α , among others. This network of eosinophil-recruiting chemokines coordinates both the ingress of eosinophils into the tissues as well as many other features of the immune response, including recruitment and activation of lymphocytes, basophils, macrophages, and neutrophils. Because of this specificity of effect of eotaxin and eotaxin-2, this pair of chemokines was investigated in an HDM model of allergic airway disease/asthma. The expression of both chemokines was abrogated in the absence of RAGE. This implies that RAGE's effect on eosinophilia is not a direct one (i.e. such that RAGE

expressed on eosinophils acts as a chemokine receptor, as has been suggested by others,⁶⁰ or that pulmonary RAGE binds surface-expressed RAGE ligands on eosinophils to assist their trafficking into the lung), but rather is mediated via the canonical cytokine-mediated pathways of eosinophil recruitment.

To address the possibility of a local effect of pulmonary epithelial RAGE on secretion of eotaxins, the antecedent link in the chain of causality, collectively the Th2 cytokines IL-5 and IL-13, was assessed. A pivotal role for RAGE in the induction of these two cytokines was demonstrated. IL-5 is known to drive many aspects of eosinophil generation, maturation, function, and survival; IL-13 mediates many of the structural changes in the lung, such as mucus hypersecretion and goblet cell hyperplasia, as well as induction of eotaxins. The finding that synthesis of IL-5 and IL-13 is abolished in RAGE's absence is thus consistent with the physiologic and histologic analyses of phenotype previously described. However, this also invokes difficult questions regarding the integrity of the IL-4 response in the absence of RAGE, and how this may be understood given the lack of IL-5 and IL-13 expression. Clearly, the absence of signaling along the RAGE axis does not eventuate in global suppression of Th2 immunity, as would be expected in the absence of the Th2 transcription factor GATA-3, or in the context of immunosuppressive therapy. Of course, this presupposes that classic T lymphocytes are indeed the major source of IL-5 and IL-13 in the asthmatic lung, and, conversely, that non-T cell sources do not account for much of the IL-4 detected in BALF. Apart from the trivial explanation of T cells expressing IL-4 but not IL-5 and IL-13 in the absence of RAGE, one potential resolution of this conundrum is the postulation of a substantially more significant role for non-classic (non T-cell) sources of IL-4, IL-5, and IL-13 than hitherto recognized.

The fundamental sub-cellular mechanisms by which IL-4 and IL-5/IL-13 secretion are decoupled are beyond the scope of this discussion. While each of these cytokines has multiple, overlapping, and synergistic effects with other Th2 and non-Th2 cytokines, it is worth noting that IL-4 has a pivotal role in antibody isotype class switching and T cell priming, which thus links this cytokine inextricably with the adaptive immune system. In contrast, in allergic airway disease/asthma, IL-5 and IL-13 appear to be primarily involved in shaping the eosinophilic inflammatory response and mediating changes in airway structure and function, respectively. Furthermore, they are not critical for T cell priming. Therefore, these two cytokines mediate the two major pre-adaptive arms of anti-microbial defense: innate immunity (i.e. the eosinophil granulocyte response) and physical barriers to pathogen entry and colonization (i.e. augmented mucus secretion to assist in expulsion of parasites). In light of this, it is not surprising that it has previously been noted by others that IL-4 production is regulated specifically by the cyclosporine-sensitive T cell receptor (TCR)/calcineurin/NFAT pathway.³³¹⁻³³³ By contrast, the calcineurin/NFAT pathway is not required for IL-5 and IL-13 induction, which rely on other signaling pathways, such as the p38 MAPK and NF- κ B pathways.³³³ The encoding of IL-5 and IL-13 regulation in these latter pathways (which are ubiquitous, not restricted to T cells, and are in fact linked to RAGE¹⁴) suggests that other cell types could be responsible for IL-5 and IL-13 production. Indeed, a recently-identified major source of IL-5 and IL-13, but not IL-4, are the nuocytes, an innate helper T cell population that resides in the normal lung, primed for rapid responses to pathogenic stimuli before T cell priming has occurred.

From a phenomenological point of view, questions proceeding from the apparent discoordination amongst the Th2 cytokines IL-4, IL-5, and IL-13, may be addressed in part by characterizing the expression of upstream cytokines and intracellular mediators. However,

fundamental mechanistic insight will likely require full dissection of the lung and draining lymphoid tissues into component cell populations and subsequent characterization of their RAGE expression status and signaling capacity, profiling of their cytokine synthetic capacity, and *in vitro* experimentation to parse out the underlying intracellular signaling pathways involved.

5.5 RAGE AND STROMA-DERIVED CYTOKINES IN ASTHMA

Stroma-derived cytokines in allergic airway disease/asthma have been the subject of intense scrutiny over the past several years. As previously noted, this is largely rooted in the presently poor understanding of the etiology of allergic disease. Each of the emerging triad of stroma-derived cytokines was evaluated in an HDM model of chronic allergic airway disease/asthma. Thymic stromal lymphopoietin (TSLP) was not found to demonstrate increased expression in response to allergen in either strain, suggesting that either the detection methods used were insufficiently sensitive or specific, or that TSLP does not play a significant role in the chronic phase of the disease. By contrast, allergen-driven expression of IL-25 is intact in the absence of RAGE. One possible explanation of this effect is that RAGE is involved in the recognition and response to IL-25, either directly or via one of the downstream mediators of the latter. Another is that endogenous IL-25 is insufficient to effect eosinophilic inflammation in the absence of one or more co-factors (which are disrupted in RAGE's absence). Although prior studies convincingly demonstrate that Th2 inflammation is blunted in IL-25 knockout mice,^{334,335} these do no more than suggest that IL-25 is a permissive factor in the allergic immune response. Hence, studies demonstrating marked eosinophilic inflammation in response to potentially supraphysiologic

exogenous IL-25^{250,253} may be of limited relevance to understanding the role of IL-25 in more physiologic models of allergic airway disease/asthma as well as the human condition itself: high doses of exogenous IL-25 may suffice to drive Th2 immunity in the absence of co-factors otherwise important in initiating allergic disease. Questions regarding the role of IL-25 in the absence of RAGE may be addressed by administering increasing doses of IL-25 to wild type and RAGE KO mice and evaluating the eosinophilic inflammatory response: demonstration of a markedly diminished response in RAGE KO mice would suggest that RAGE lies downstream of IL-25, whereas similar responses in both strains would suggest a non-physiologic effect of exogenous IL-25 and would indicate that endogenous allergen-elicited IL-25 is permissive but not sufficient for the development of a Th2 immune response.

Characterization of IL-33 expression in response to HDM allergen in wild type and RAGE KO mice was of particular importance because IL-33 has previously been linked to specific induction of IL-5 and IL-13 (but not IL-4). This complements the finding that absence of RAGE prevents induction of specifically IL-5 and IL-13 (but not IL-4) in response to allergen. Because IL-33 is constitutively expressed in the interior of structural cells of the lung (in which it serves as a transcription repressor), detection of released BALF was attempted in order to restrict the scope of investigation solely to the portion of IL-33 that acts as an extracellular cytokine. Release of IL-33 into BALF was not observed in a chronic HDM model of allergic airway disease/asthma, nor acutely following single dose administration of HDM extract. Although no IL-33 could be detected in either instance, it is possible that this was the case because of a narrow time window during which IL-33 secretion is appreciable in response to stimulus.

The increase in total IL-33 at both transcript and protein levels in wild type mice treated with allergen is consistent with increased turnover of IL-33 from intracellular stores and shunting

into secretory pathways. As IL-33 has an additional role in regulating transcription, tight control of IL-33 levels, such that steady state is maintained, is critical (with synthesis, regulated secretion in response to allergen stimulus, and transcriptional regulation in the balance). Up-regulation in global IL-33 expression could be attributed to recruitment of novel cell populations into the pool of IL-33-expressing cells, proliferation of established IL-33-expressing cells, or increased mean IL-33 expression on a per cell basis. The former two scenarios would be compatible with a bystander increase in the levels of IL-33, perhaps induced by the inflammatory state in the lungs of wild type mice; the latter scenario would be most consistent with compensatory up-regulation of IL-33 release due to induced secretion. Immunofluorescence microscopy studies suggest that IL-33 is increased in wild type mice in response to HDM extract on a per cell basis, lending support to the notion that allergen induces enhanced IL-33 synthesis and secretion from structural cells of the lung. RAGE KO mice do not demonstrate enhanced IL-33 expression in response to allergen, suggesting that the abrogation of IL-5 and IL-13 expression may be attributed to a deficient IL-33 response in RAGE's absence.

As in the case of IL-25, it is unclear whether RAGE lies upstream or downstream of IL-33. Conceivable mechanistic scenarios include but are not limited to: RAGE signaling induces the expression and secretion of IL-33, RAGE acts as a receptor or co-receptor for IL-33 on responding immune cells, and RAGE is involved in recruiting IL-33 responsive cells, such as nuocytes, to the lung compartment. The latter two hypothetical mechanisms would require RAGE to take part in an autoregulatory positive feedback loop driving reciprocal IL-33 synthesis and secretion; in the absence of RAGE, this loop would be broken and thus RAGE KO mice would not demonstrate elevated IL-33 in response to allergen. The first of the proposed mechanisms, that of RAGE inducing IL-33, is the simplest and most attractive: the lack of

elevated allergen-induced IL-33 expression in the absence of RAGE easily explains the downstream effects on IL-5, IL-13, airway physiology, and pulmonary histology seen in RAGE KO mice administered allergen. The observation that the major source of IL-33 in mouse lung appears to be the type II pneumocyte, in structural and functional proximity to and lineage antecedent of the major source of RAGE (the type I pneumocyte), further supports a mechanism in which alveolar epithelial RAGE signaling promotes the release of IL-33 from neighboring alveolar epithelium, which in turn drives downstream cytokine expression. Questions concerning the place of IL-33 in the chain of causality may be readily addressed by administration of exogenous IL-33 in RAGE KO mice in a dosing regimen known to elicit pulmonary eosinophilic inflammation in wild type mice. If RAGE KO mice reproduce this inflammation, it would be reasonable to conclude that RAGE is an inducer of IL-33, and that IL-33 sensing and response systems are intact; if the inflammatory response is blunted in RAGE's absence, a mechanism in which RAGE is involved in detection and response to IL-33 (as a receptor, a co-receptor, or as a more distant permissive factor) would be more likely.

5.6 SUMMARY AND FUTURE DIRECTIONS

The results presented herein suggest a vital role for RAGE in the pathogenesis of allergic airway disease/asthma. What emerges is a complex picture of mixed phenotype, in which the absence of RAGE abolishes allergen-induced eosinophilic inflammation, mucus hypersecretion, and airway hyperresponsiveness, even as the immunoglobulin response remains fully intact. These phenotypic data are paralleled by the cytokine profile, with IL-5, IL-13, and eotaxins linked to inflammation, airway physiology, and airway structure, and IL-4 linked to immunoglobulin.

Investigation of stroma-derived cytokines demonstrated no role for TSLP in the animal models studied and no difference between wild type and RAGE KO mice with respect to IL-25 secretion. IL-33, a potent inducer of IL-5 and IL-13 (but not IL-4) was found to have disrupted regulation in response to allergen in RAGE's absence, consistent with the phenotypic and overall cytokine data. Given the fact that RAGE demonstrates the same essential role in the generation of Th2 immunity in mouse models of asthma with markedly different antigens (HDM extract vs. ovalbumin) and routes of sensitization, and given the very low affinity of RAGE for proteins present in HDM extract, it seems likely that designated endogenous RAGE ligands serve to link exogenous allergens and their associated danger signals with signaling pathways downstream from RAGE.

On the basis of these studies and in conjunction with observations made by others, a working model of RAGE function in allergic airway disease/asthma emerges. Allergens and their associated danger signals provoke the release of one or more RAGE ligands from one or more cell sources in the lung. With reference to this, it is important to note that ovalbumin requires traces of LPS to have adequate immunogenicity³³⁶ and that protease activity of HDM extract is crucial for development of the full asthmatic phenotype.³³⁷⁻³³⁹ Interestingly, one study also suggested that TLR4 signaling is vital in inducing disease in an HDM model of asthma.³²¹ This suggests that, while foreign antigen may suffice to drive immunoglobulin production, associated danger signals (e.g. LPS together with T cell-mediated damage in the ovalbumin model or protease-mediated necrosis in the HDM model) are required for the development of a mature Th2 immune response. Indeed, the requirement for LPS as well as tissue damage (whether by allergen proteases in the HDM model or cytotoxic T lymphocytes and antibody-mediated complement deposition in the ovalbumin model) may suggest a two-pronged initiation of the

fully-fledged Th2 immune response: canonical PAMPS, such as LPS, drive T cell priming and differentiation and B cell antibody secretion, whereas low-level tissue damage and elaboration of specific endogenous DAMPS skews the lymphocyte response towards specifically Th2. Following endogenous RAGE ligand release, said ligands activate RAGE on type I alveolar epithelium and/or other cell types. Although the compartment and specific cell population that expresses RAGE critical to the phenotype seen in allergic airway disease/asthma was never definitively identified, for purposes of this discussion it is assumed that this is lung and specifically type I alveolar epithelial cells. The activated RAGE-expressing cells then induce IL-33-expressing cells to release that cytokine and also up-regulate basal levels of stored IL-33. This is likely mediated by autocrine, paracrine, or juxtacrine signaling: for example, type I pneumocytes may signal to neighboring type II pneumocytes via receptor-membrane ligand interactions, gap junctions, or secreted factors. Released IL-33, in turn, may then act on the resident innate nuocytes and recruited T lymphocytes to potently induce IL-5 and IL-13 secretion, which eventuates in most of the major manifestations of allergic airway disease/asthma. This is summarized graphically below (Figure 45).

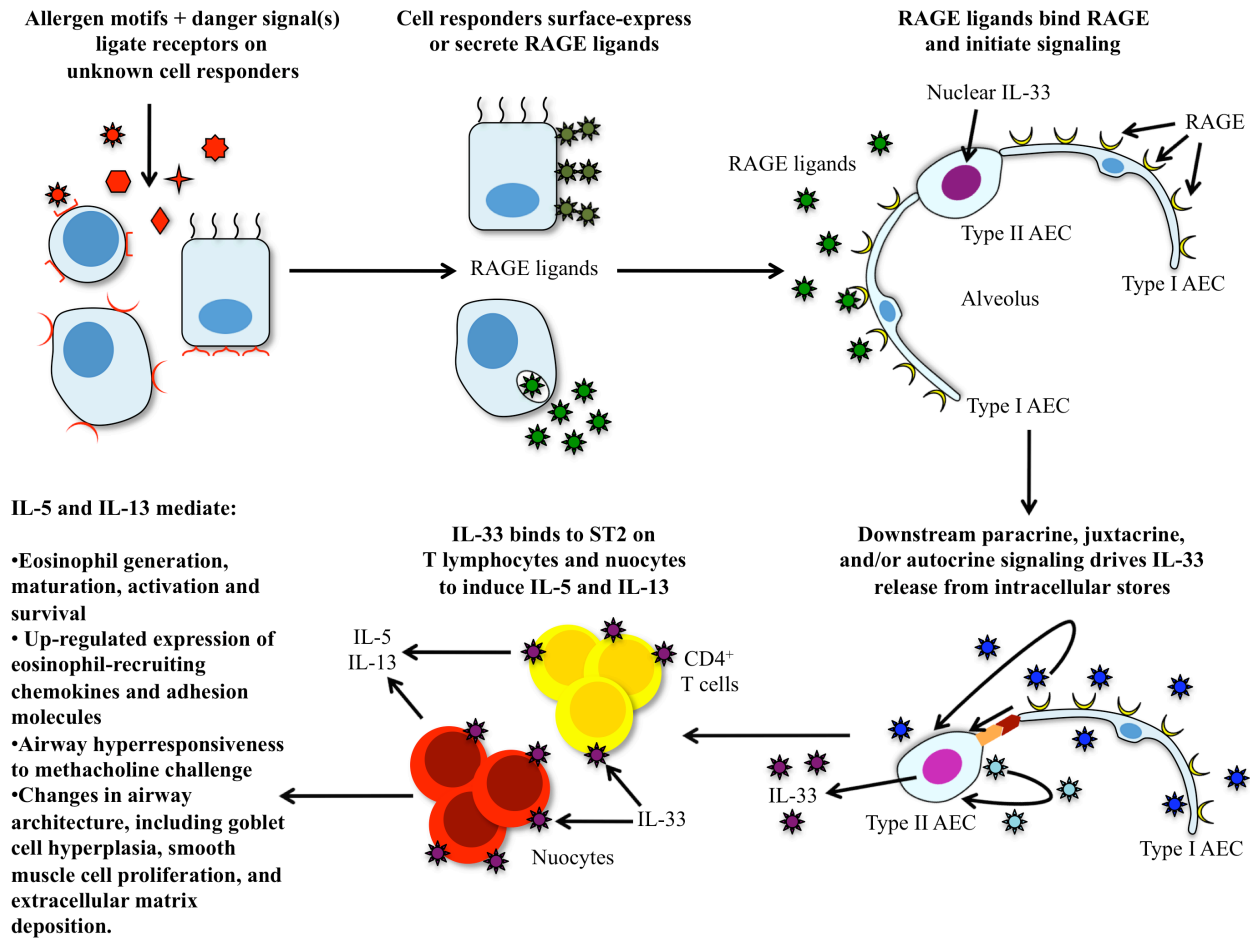


Figure 45. A schematic representation depicting a model of the role of RAGE in asthma.

Allergen along with danger signals elicits release of endogenous RAGE ligands, which then activate signaling cascades in RAGE-expressing cells. Activated RAGE-expressing cells, in turn, induce IL-33-expressing cells to up-regulate and release IL-33. Released IL-33 acts on responding cells such as nuocytes and T lymphocytes to drive expression of IL-5 and IL-13, resulting in physiologic and structural changes in lung consistent with asthma.

These studies suggest many new questions and avenues of future investigation. The most immediate set of questions regards the role of other cytokines, chemokines, and adhesion molecules in animal models of asthma, including but not limited to IL-9 expression, Th1 and other Th17 cytokines, the PSGL-1/P-selectin interaction, and eosinophil chemotaxins other than eotaxin and eotaxin-2. As the stroma-derived cytokine TSLP appears to have been irrelevant in the chronic HDM model of allergic airway disease/asthma assessed, implementation of more

acute models to assess the role of RAGE in the regulation of this cytokine may be necessary. Methods to assess IL-33 secretion specifically (rather than whole tissue levels), perhaps by use of *ex vivo* explants of lung, are required to demonstrate that not only basal expression but also secretion of IL-33 in response to allergen stimulus is impaired in RAGE's absence. Furthermore, the role of RAGE in IL-25 and especially IL-33 function is somewhat murky and must be clarified with administration of cytokines to wild type and RAGE KO mice and assessment of inflammatory phenotype. If RAGE KO mice demonstrate an impaired inflammatory response to IL-25 and/or IL-33, it is reasonable to conclude that RAGE lies downstream of these cytokines (e.g. as a receptor, co-receptor, nuocyte maintenance factor, etc.). Contrariwise, if RAGE KO mice demonstrate similar inflammation in response to IL-25 and/or IL-33 as their wild type counterparts, RAGE being positioned functionally antecedent to these cytokines is the more likely possibility.

A second line of potential investigation concerns unresolved questions regarding localization of RAGE and Th2 cytokine expression. Bone marrow chimeric studies failed to demonstrate whether pulmonary or hematopoietic RAGE is responsible for the phenotypic effects seen in RAGE KO mice. This may be addressed by tissue-specific knockout mice, in which hematopoietic or pulmonary RAGE is selectively eliminated from the system. Additionally, whole lung single cell suspension followed by immunostaining for lineage markers and separation of the various populations by flow cytometry would permit rigorous assessment of RAGE and cytokine expression status, via culturing and subsequent ELISA on conditioned media, intracellular cytokine staining, qRT-PCR on extracted RNA, and/or immunoblotting on purified cell lysates. In particular, the IL-33-expressing population chiefly responsible for IL-33 secretion in response to HDM allergen is unclear, i.e. type II pneumocytes vs. smooth muscle

cells; furthermore, the respective roles of T lymphocytes vs. non-T cell sources such as nuocytes in the global expression of IL-5 and IL-13 in animal models of asthma is unclear and requires elucidation via the preparative and analytical approaches discussed previously.

A third set of questions concerns the signal transduction mechanism between allergen and RAGE. Affinity chromatography approaches to detecting endogenous RAGE ligands generated numerous identifications, but their biological significance remains unclear. A more physiologic approach to ligand identification is needed. One approach is to use sRAGE conjugated to a functionalized photoactivatable cross-linker to probe for RAGE ligands. Whole lung single cell suspensions from mice previously sensitized and challenged with HDM extract or saline vehicle control could be prepared and cultured in serum-free media, with or without HDM extract. sRAGE conjugated to photoactivatable cross-linker functionalized with a fluorophore (such as sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamido)ethyl-1,3'-dithiopropionate (sulfo-SAED)) could then be introduced to the whole lung cell culture, under non-permeabilizing or membrane permeabilizing conditions. Non-permeabilizing conditions would assess for surface-expressed RAGE ligands, whereas saponin-mediated membrane permeabilization (perhaps in conjunction with application of brefeldin A, to block secretion) would assess for secreted or intracellular RAGE ligands. Non-covalent sRAGE interactions with cognate ligands would be rendered covalent via photoactivation of the cross-linker with exposure to UV light. Cell sorting on the fluorescently labeled cells (with appropriate controls for nonspecific binding) or inspection by immunofluorescence microscopy would indicate the major cellular sources of RAGE ligands. With this information in hand, these *in vitro* experiments could be repeated in lung cells pre-sorted according to unique lineage markers (based on RAGE ligand expression, determined previously), using sRAGE conjugated to a biotinylated non-fluorescent cross-linker

(such as sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido) hexanoamido]ethyl-1,3'-dithiopropionate) (sulfo-SBED)). Identification of RAGE ligands in various cell types could be made by isolation of biotinylated species from cell lysates using solid phase streptavidin, followed by release of the sRAGE/RAGE ligand complexes from streptavidin-bound biotin by reduction of disulfide bonds, separation by SDS-PAGE, and mass spectrometry. Determination of physiologic relevance could be made by quantitative proteomics (perhaps by using methods such as two-dimensional difference gel electrophoresis (DIGE)), comparing HDM extract-treated samples with saline-treated samples to confirm up-regulation of putative ligands in the allergic state. Further corroboration of relevance may be attained by expression profiling of identified RAGE ligands using standard immunodetection methods and through use of anti-ligand antagonistic antibodies in animal models of allergic airway disease/asthma to assess the effect on disease phenotype and cytokine expression.

A fourth line of inquiry concerns the signal transduction mechanism that connects RAGE to IL-33 synthesis and secretion. Elucidation would mandate characterization of physiologic RAGE ligands involved in allergy, identification of the source(s) of RAGE (e.g. type I alveolar epithelial cells or alveolar macrophages, etc.) relevant to the phenotypic effects seen in animal models of disease, as well as identification of the cell type(s) whose allergen-induced up-regulation and release of IL-33 is aberrantly regulated in RAGE's absence (e.g. type II alveolar epithelial cells or smooth muscle cells). With these data available, *in vitro* experiments may be devised that explore the relationship between RAGE and IL-33 in detail. For instance, if it appears that type I pneumocyte RAGE and type II pneumocyte IL-33 are most relevant to the effects seen, and that S100A13 is the dominant RAGE ligand involved, studies employing primary mixed co-cultures of type I and type II epithelial cells (with S100A13 used as an

agonist) may be used to probe the intra- and intercellular networks mediating RAGE's effect on IL-33. Using RAGE agonist as the input and IL-33 secretion as the output, small molecule inhibitors of surface receptors or gap junctions, or antagonistic antibodies against cell surface adhesion molecules and secreted proteins, could be used to parse out the most relevant autocrine, paracrine, and juxtacrine interactions. Once these are delineated, additional experiment may be performed on cultures of a single cell population to characterize the intracellular signaling cascades linking RAGE ligation to downstream cell responses and, separately, IL-33 secretion to upstream extracellular stimuli.

Laying aside mechanistic questions, findings regarding the role of RAGE in animal models of asthma may have substantial bearing on other models of disease. First, results in the ovalbumin and HDM extract models of asthma may be confirmed using other antigens (e.g. cockroach feces) and other strains (e.g. BALB/c). Furthermore, RAGE's role in the early phase of allergic airway disease/asthma must be elucidated using more acute models of disease. Second, it was observed that wild type, but not RAGE KO, mice treated with HDM extract develop prominent vascular smooth muscle hyperplasia, a histologic phenotype remarkably similar to that seen in pulmonary hypertension. Although smooth muscle hyperplasia may represent merely a reaction to the inflammatory milieu in the lung, it is worth exploring the feasibility of using HDM extract to develop mouse models of pulmonary hypertension, especially as RAGE has recently been found to be critically involved in vascular remodeling.³⁴⁰ Finally, RAGE's prominent role in the maladaptive allergic response suggests that it may be involved in the adaptive response to helminth infection. Indeed, from the pharmacologic standpoint, RAGE affinity for glycans makes it a prime candidate for a pattern recognition receptor for parasites and helminths, which are rich in glycoproteins and glycolipids and whose

glycans are increasingly recognized as profoundly immunomodulatory.³⁴¹⁻³⁴³ Investigation of the role of RAGE in animal models of nematode (e.g. *Nippostrongylus*) and trematode (e.g. *Schistosoma*) infection would address some of these questions.

It must be stated that these studies of the role of RAGE in asthma were conducted exclusively using mouse models of allergic airway disease/asthma, with well-controlled application of allergen and inbred mouse strains. The human disease is far more complex, with substantial genetic heterogeneity, varied environmental triggers, and variable phenotype and responsiveness to treatment. Bearing in mind these not uncommon discrepancies between animal disease models and the human conditions they attempt to model, the studies conducted thus far strongly suggest that RAGE is a potential therapeutic target in the treatment of asthma. Small molecule RAGE inhibitors, such as the recently developed compound TTP488 (already in clinical trials in Alzheimer's disease patients), may be tested in mice to lend further support to the conclusions drawn from the sRAGE therapeutic studies, as well as to assess for possible side effects of RAGE inhibition. Use of such inhibitors would also provide an answer to the question of whether RAGE inhibition in frank allergic airway disease/asthma has the capacity to ameliorate disease phenotype (to test this, anti-RAGE therapy would commence after allergic airway disease was fully established). Finally, confirmatory studies on human lung tissue explants, primary culture cells, and on BALF and serum samples will be necessary to establish the degree to which the results in mice apply in humans. With regard to this, the elucidation of the signaling circuits by which a disease process initiating in the distal alveolus progresses proximally to result in a predominantly bronchial disease will constitute an important aim of future research.

APPENDIX

Table 1. RAGE binding partners detected in HDM extract, identified by mass spectrometry.

Accession No.	Protein Name	Mass kDa	Score	Coverage %	emPAI
gi 49066042	RecName: Full=Calmodulin-alpha; Short=CaM A	16.037	1597	23.2	0.92
gi 2353266	tropomyosin [<i>Dermatophagoides pteronyssinus</i>]	32.953	1482	49.3	4.08
gi 162532787	calmodulin 1 [<i>Crassostrea ariakensis</i>]	5.657	232	75	0.76
gi 196476746	calmodulin 2 [<i>Amblyomma americanum</i>]	4.294	210	42.1	3.15
gi 136050	RecName: Full=Troponin C	17.422	190	20.3	0.22
gi 308476320	hypothetical protein CRE_18007 [<i>Caenorhabditis remanei</i>]	41.642	149	5.6	0.19
gi 62825408	calmodulin [<i>Orthopyxis integra</i>]	11.94	127	32.7	0.77
gi 20385544	group 14 allergen protein [<i>Dermatophagoides pteronyssinus</i>]	191.455	122	3.1	0.06
gi 219815476	troponin C [<i>Tyrophagus putrescentiae</i>]	17.68	91	15	0.22
gi 156336944	hypothetical protein NEMVEDRAFT_v1g150407 [<i>Nematostella vectensis</i>]	7.062	79	29.7	0.59
gi 15072346	ferritin heavy chain-like protein [<i>Dermatophagoides pteronyssinus</i>]	20.875	65	5.6	0.18

Table 1 (continued).

gi 48112268	PREDICTED: hypothetical protein LOC409648 [<i>Apis mellifera</i>]	22.995	63	4	0.17
gi 2347119	thioredoxin peroxidase [<i>Dirofilaria immitis</i>]	22.336	61	5.5	0.17
gi 241999902	bridging integrator, putative [<i>Ixodes scapularis</i>]	75.409	58	1.7	0.05
gi 321462817	hypothetical protein DAPPUDRAFT_215354 [<i>Daphnia pulex</i>]	61.061	56	1.7	0.06

Table 2. RAGE binding partners, identified by mass spectrometry, detected in lung homogenates from WT or RAGE KO mice treated with HDM extract or saline control.

Accession No.	Protein Name	Mass kDa	Score	Coverage %	emPAI
WT, saline-treated					
P43277	Histone H1.3	22.1	3015	28.1	5.8
P43274	Histone H1.4	21.977	2928	28.3	5.87
P43276	Histone H1.5	22.576	1248	27.4	3.1
P09405	Nucleolin	76.723	1086	16.1	0.76
Q9CZX8	40S ribosomal protein S19	16.085	907	37.9	3.55
Q8CGP5	Histone H2A type 1-F	14.162	845	15.4	1.08
P67984	60S ribosomal protein L22	14.759	795	28.9	1.56

Table 2 (continued).

P28653	Biglycan	41.639	761	9.2	0.41
P60710	Actin, cytoplasmic 1	41.737	738	14.1	0.53
Q64475	Histone H2B type 1-B	13.952	730	33.3	1.69
P43275	Histone H1.1	21.785	728	17.4	1.25
P10922	Histone H1.0	20.861	486	16	0.96
P62751	60S ribosomal protein L23a	17.695	476	16	0.81
P62852	40S ribosomal protein S25	13.742	438	26.4	1.12
Q6Z WV7	60S ribosomal protein L35	14.553	436	22	1.59
A2ASQ1	Agrin	207.539	428	2.9	0.07
P62889	60S ribosomal protein L30	12.784	383	31.3	1.22
Q62151	Advanced glycosylation end product-specific receptor	42.669	331	3.5	0.18
P28654	Decorin	39.809	322	12.1	0.43
P62806	Histone H4	11.367	303	29.1	1.47
P97352	Protein S100-A13	11.158	299	19.4	0.85
Q05793	Basement membrane-specific heparan sulfate proteoglycan core protein	398.294	299	1.6	0.05
O54724	Polymerase I and transcript release factor	43.954	287	6.6	0.39

Table 2 (continued).

P68433	Histone H3.1	15.404	252	13.2	0.56
P63017	Heat shock cognate 71 kDa protein	70.871	246	6	0.17
Q8VCC9	Spondin-1	90.821	227	3.7	0.12
Q9JHL1	Na(+)/H(+) exchange regulatory cofactor NHE-RF2	37.403	204	8	0.21
Q8VCT4	Carboxylesterase 3	61.788	202	4.8	0.19
P62900	60S ribosomal protein L31	14.463	194	16.8	1.05
P62204	Calmodulin	16.838	185	15.4	0.51
P20029	78 kDa glucose-regulated protein	72.422	175	3.5	0.11
Q8BP67	60S ribosomal protein L24	17.779	174	8.3	0.22
P62264	40S ribosomal protein S14	16.273	174	15.9	0.53
P10649	Glutathione S-transferase Mu 1	25.97	171	13.8	0.5
P47738	Aldehyde dehydrogenase, mitochondrial	56.538	171	3.7	0.13
O08553	Dihydropyrimidinase-related protein 2	62.278	168	5.2	0.19
P62862	40S ribosomal protein S30	6.648	168	16.9	0.63
Q9CR57	60S ribosomal protein L14	23.564	168	6	0.16
P68372	Tubulin beta-2C chain	49.831	165	8.3	0.24

Table 2 (continued).

P61255	60S ribosomal protein L26	17.258	164	17.2	0.84
P15532	Nucleoside diphosphate kinase A	17.208	161	17.1	0.5
P29341	Polyadenylate-binding protein 1	70.671	159	3.5	0.11
P62082	40S ribosomal protein S7	22.127	155	18	0.89
Q63918	Serum deprivation-response protein	46.764	153	6.9	0.26
P97351	40S ribosomal protein S3a	29.885	146	7.6	0.27
P47911	60S ribosomal protein L6	33.51	146	9.5	0.38
P50404	Pulmonary surfactant-associated protein D	37.688	145	4.8	0.21
P62908	40S ribosomal protein S3	26.674	137	12.8	0.49
P62320	Small nuclear ribonucleoprotein Sm D3	13.916	130	15.1	0.64
P10126	Elongation factor 1-alpha 1	50.114	128	2.4	0.07
P62141	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	37.187	126	3.1	0.1
Q62093	Serine/arginine-rich splicing factor 2	25.476	125	3.6	0.15
A2AJK6	Chromodomain-helicase-DNA-binding protein 7	334.061	116	0.2	0.01
P12970	60S ribosomal protein L7a	29.977	115	10.5	0.43
P97315	Cysteine and glycine-rich protein 1	20.583	114	7.8	0.18

Table 2 (continued).

P24549	Retinal dehydrogenase 1	54.468	114	5	0.22
P61358	60S ribosomal protein L27	15.798	112	5.9	0.25
Q8CCK0	Core histone macro-H2A.2	40.092	111	2.4	0.09
Q8CI43	Myosin light chain 6B	22.749	105	6.3	0.17
O35381	Acidic leucine-rich nuclear phosphoprotein 32 family member A	28.538	105	9.7	0.45
P32067	Lupus La protein homolog	47.756	103	2.4	0.08
P30115	Glutathione S-transferase A3	25.361	100	7.2	0.32
P47915	60S ribosomal protein L29	17.587	98	6.9	0.22
P39447	Tight junction protein ZO-1	194.742	96	0.6	0.02
P01942	Hemoglobin subunit alpha	15.085	95	16.9	0.58
Q9JJH1	Ribonuclease 4	17.025	90	7.4	0.22
P97429	Annexin A4	35.916	88	6.3	0.22
P84099	60S ribosomal protein L19	23.466	86	4.6	0.16
P62242	40S ribosomal protein S8	24.205	86	6.3	0.16
P14069	Protein S100-A6	10.051	85	9	0.4
P20152	Vimentin	53.688	85	8.6	0.22

Table 2 (continued).

P26041	Moesin	67.767	85	2.9	0.11
P62270	40S ribosomal protein S18	17.719	79	13.2	0.48
P05064	Fructose-bisphosphate aldolase A	39.356	78	3	0.09
P16858	Glyceraldehyde-3-phosphate dehydrogenase	35.81	77	4.5	0.1
P70441	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	38.6	76	3.1	0.1
Q9Z204	Heterogeneous nuclear ribonucleoproteins C1/C2	34.385	75	6.1	0.23
Q8BH43	Wiskott-Aldrich syndrome protein family member 2	54.074	75	3.6	0.14
Q61937	Nucleophosmin	32.56	74	4.5	0.12
P63101	14-3-3 protein zeta/delta	27.771	74	5.7	0.14
Q9EQU5	Protein SET	33.378	73	3.5	0.11
Q6IFZ6	Keratin, type II cytoskeletal 1b	61.359	72	2.1	0.06
P48036	Annexin A5	35.752	72	6.6	0.22
P26040	Ezrin	69.407	71	2.9	0.11
Q62376	U1 small nuclear ribonucleoprotein 70 kDa	51.992	69	1.6	0.07
Q06318	Uteroglobin	10.519	67	10.4	0.37
P35979	60S ribosomal protein L12	17.805	67	5.5	0.21

Table 2 (continued).

Q62465	Synaptic vesicle membrane protein VAT-1 homolog	43.097	66	4.9	0.18
P11247	Myeloperoxidase	81.182	66	1.3	0.05
P35242	Pulmonary surfactant-associated protein A	26.157	63	5.2	0.14
Q7TMK9	Heterogeneous nuclear ribonucleoprotein Q	69.633	62	1.3	0.05
Q60972	Histone-binding protein RBBP4	47.656	62	1.6	0.08
Q9JJW6	RNA and export factor-binding protein 2	23.73	61	5	0.16
Q03265	ATP synthase subunit alpha, mitochondrial	59.753	61	1.8	0.06
Q3TEA8	Heterochromatin protein 1-binding protein 3	60.867	61	3.4	0.13
Q9CQI7	U2 small nuclear ribonucleoprotein B"	25.323	59	3.6	0.15
Q8C119	Protein NDNF	65.027	59	1.9	0.06
P97447	Four and a half LIM domains protein 1	31.889	59	3.9	0.11
Q9CYR0	Single-stranded DNA-binding protein, mitochondrial	17.319	59	5.3	0.22
P40936	Indolethylamine N- methyltransferase	29.46	57	4.2	0.13
P11499	Heat shock protein HSP 90-beta	83.281	57	1.4	0.04
P62918	60S ribosomal protein L8	28.025	55	4.3	0.13
Q60737	Casein kinase II subunit alpha	45.134	54	1.8	0.08

Table 2 (continued).

O08709	Peroxiredoxin-6	24.871	52	8	0.33
Q9Z1P7	KN motif and ankyrin repeat domain-containing protein 3	84.186	52	2.3	0.09
P62702	40S ribosomal protein S4, X isoform	29.598	51	2.7	0.13
P62754	40S ribosomal protein S6	28.681	50	3.2	0.13
Q9DCW4	Electron transfer flavoprotein subunit beta	27.623	48	4.3	0.14
P97822	Acidic leucine-rich nuclear phosphoprotein 32 family member E	29.622	48	5.4	0.13
P08074	Carbonyl reductase [NADPH] 2	25.958	47	4.5	0.15
P58252	Elongation factor 2	95.314	46	1	0.04
Q8VHE6	Dynein heavy chain 5, axonemal	527.558	46	0.2	0.01
P40630	Transcription factor A, mitochondrial	27.988	46	4.5	0.13
P17563	Selenium-binding protein 1	52.514	46	2.3	0.07
P47962	60S ribosomal protein L5	34.401	45	4.4	0.11
Q9WVE8	Protein kinase C and casein kinase substrate in neurons protein 2	55.833	45	1.9	0.07
Q9CXW4	60S ribosomal protein L11	20.252	44	5.1	0.19
P60867	40S ribosomal protein S20	13.373	44	5.9	0.29
P70279	Surfeit locus protein 6	41.235	43	2.3	0.09

Table 2 (continued).					
Q99K48	Non-POU domain-containing octamer-binding protein	54.541	43	1.7	0.07
P07724	Serum albumin	68.693	43	1.6	0.05
O55142	60S ribosomal protein L35a	12.554	42	6.4	0.31
Q6ZWN5	40S ribosomal protein S9	22.591	41	4.1	0.17
P27048	Small nuclear ribonucleoprotein-associated protein B	23.656	40	3	0.16
WT, HDM extract-treated					
P09405	Nucleolin	76.723	1804	18	0.76
Q64475	Histone H2B type 1-B	13.952	1192	31.7	1.69
P28653	Biglycan	41.639	1064	19.5	0.82
P62204	Calmodulin	16.838	1031	55	1.29
Q62151	Advanced glycosylation end product-specific receptor	42.669	966	24.6	0.52
Q8CGP5	Histone H2A type 1-F	14.162	924	21.5	0.63
P67984	60S ribosomal protein L22	14.759	923	49.2	2.24
P62082	40S ribosomal protein S7	22.127	828	29.9	1.61
Q9CZX8	40S ribosomal protein S19	16.085	786	40	3.55
P43274	Histone H1.4	21.977	732	21.9	1.23

Table 2 (continued).

P62751	60S ribosomal protein L23a	17.695	704	16	0.81
P43277	Histone H1.3	22.1	688	21.7	1.22
Q61937	Nucleophosmin	32.56	460	12.3	0.39
P62889	60S ribosomal protein L30	12.784	424	31.3	0.7
P62737	Actin, aortic smooth muscle	42.009	385	9.3	0.18
P99024	Tubulin beta-5 chain	49.671	366	9.9	0.33
P68372	Tubulin beta-2C chain	49.831	338	9.9	0.33
P62852	40S ribosomal protein S25	13.742	333	20.8	1.12
P32067	Lupus La protein homolog	47.756	324	5.8	0.16
P62320	Small nuclear ribonucleoprotein Sm D3	13.916	301	15.1	0.64
O54724	Polymerase I and transcript release factor	43.954	294	13.3	0.28
P16254	Signal recognition particle 14 kDa protein	12.51	288	12.7	0.31
P16110	Galectin-3	27.515	282	5.7	0.14
Q922U2	Keratin, type II cytoskeletal 5	61.767	282	2.1	0.06
P62900	60S ribosomal protein L31	14.463	253	21.6	1.05
P62309	Small nuclear ribonucleoprotein G	8.496	251	17.1	0.48

Table 2 (continued).

P97315	Cysteine and glycine-rich protein 1	20.583	248	7.8	0.18
P62862	40S ribosomal protein S30	6.648	230	18.6	1.66
P97352	Protein S100-A13	11.158	228	17.3	0.36
Q9Z204	Heterogeneous nuclear ribonucleoproteins C1/C2	34.385	222	6.7	0.23
P62315	Small nuclear ribonucleoprotein Sm D1	13.282	211	16.8	0.3
P62305	Small nuclear ribonucleoprotein E	10.804	206	29.3	1.56
Q6ZWV7	60S ribosomal protein L35	14.553	204	22	0.61
Q8VCC9	Spondin-1	90.821	198	4.3	0.12
P24369	Peptidyl-prolyl cis-trans isomerase B	23.713	198	7.9	0.35
P62806	Histone H4	11.367	196	17.5	0.83
P27048	Small nuclear ribonucleoprotein-associated protein B	23.656	186	9.5	0.56
Q9EQU5	Protein SET	33.378	185	3.5	0.11
P26040	Ezrin	69.407	179	4.1	0.11
P68369	Tubulin alpha-1A chain	50.136	159	3.3	0.07
P97351	40S ribosomal protein S3a	29.885	152	11.7	0.43
Q8BP67	60S ribosomal protein L24	17.779	130	8.3	0.22

Table 2 (continued).

P62843	40S ribosomal protein S15	17.04	128	15.2	0.23
P28654	Decorin	39.809	124	5.9	0.2
P28656	Nucleosome assembly protein 1-like 1	45.345	120	4.3	0.08
P68433	Histone H3.1	15.404	118	5.1	0.25
P63325	40S ribosomal protein S10	18.916	114	5.5	0.2
Q60972	Histone-binding protein RBBP4	47.656	112	5.4	0.25
P63017	Heat shock cognate 71 kDa protein	70.871	108	5.3	0.11
P02104	Hemoglobin subunit epsilon-Y2	16.137	105	6.8	0.24
P10922	Histone H1.0	20.861	101	8.8	0.4
P29341	Polyadenylate-binding protein 1	70.671	98	3.5	0.05
Q9CXW4	60S ribosomal protein L11	20.252	97	7.9	0.19
P01942	Hemoglobin subunit alpha	15.085	94	6.3	0.26
Q3UPL0	Protein transport protein Sec31A	133.569	91	0.6	0.03
Q63918	Serum deprivation-response protein	46.764	87	6.2	0.17
P61255	60S ribosomal protein L26	17.258	84	10.3	0.5
P16858	Glyceraldehyde-3-phosphate dehydrogenase	35.81	79	3.6	0.1

Table 2 (continued).

A2BDX3	Adenylyltransferase and sulfurtransferase MOCS3	49.375	77	2	0.07
P10126	Elongation factor 1-alpha 1	50.114	68	2.4	0.07
P12970	60S ribosomal protein L7a	29.977	67	4.1	0.13
P30115	Glutathione S-transferase A3	25.361	65	12.2	0.15
Q62376	U1 small nuclear ribonucleoprotein 70 kDa	51.992	64	1.6	0.07
Q62093	Serine/arginine-rich splicing factor 2	25.476	62	3.6	0.15
O35381	Acidic leucine-rich nuclear phosphoprotein 32 family member A	28.538	59	2.4	0.13
P62307	Small nuclear ribonucleoprotein F	9.725	57	9.3	0.41
Q7TMK9	Heterogeneous nuclear ribonucleoprotein Q	69.633	55	1.3	0.05
P84104	Splicing factor, arginine/serine-rich 3	19.33	53	5.5	0.2
Q8BH43	Wiskott-Aldrich syndrome protein family member 2	54.074	47	1.6	0.07
Q8VEK3	Heterogeneous nuclear ribonucleoprotein U	87.918	45	0.9	0.04
P62702	40S ribosomal protein S4, X isoform	29.598	34	3.4	0.13
RAGE KO, saline-treated					
P09405	Nucleolin	76.723	2307	27.9	1.57
Q8VCC9	Spondin-1	90.821	1922	25.7	0.94

Table 2 (continued).

P43274	Histone H1.4	21.977	1912	28.3	3.98
P15864	Histone H1.2	21.267	1609	33.5	3.46
Q9CZX8	40S ribosomal protein S19	16.085	1362	57.9	7.7
P62204	Calmodulin	16.838	1213	41.6	1.29
P28653	Biglycan	41.639	1176	23	0.98
P67984	60S ribosomal protein L22	14.759	1147	40.6	2.24
P60710	Actin, cytoplasmic 1	41.737	1136	22.1	0.82
Q8R1M2	Histone H2A.J	14.045	1130	43.4	1.69
P10922	Histone H1.0	20.861	1029	34	2.86
Q6GSS7	Histone H2A type 2-A	14.095	1016	43.1	1.67
Q64475	Histone H2B type 1-B	13.952	960	43.7	6.23
P62889	60S ribosomal protein L30	12.784	857	60	9.87
P68372	Tubulin beta-2C chain	49.831	850	21.1	0.77
Q9ERD7	Tubulin beta-3 chain	50.419	776	15.8	0.53
P63017	Heat shock cognate 71 kDa protein	70.871	774	22.9	0.58
P62806	Histone H4	11.367	738	45.6	5.1

Table 2 (continued).

P43276	Histone H1.5	22.576	687	25.6	2
P62751	60S ribosomal protein L23a	17.695	616	23.7	2.28
O08583	THO complex subunit 4	26.94	501	12.5	0.48
O35381	Acidic leucine-rich nuclear phosphoprotein 32 family member A	28.538	499	15.4	1.11
Q6Z WV7	60S ribosomal protein L35	14.553	488	22	0.61
P43275	Histone H1.1	21.785	469	11.7	0.63
P62082	40S ribosomal protein S7	22.127	467	26.8	1.61
P47962	60S ribosomal protein L5	34.401	454	20.5	0.68
P62900	60S ribosomal protein L31	14.463	444	24.8	1.61
P62852	40S ribosomal protein S25	13.742	392	32	1.73
P97352	Protein S100-A13	11.158	372	19.4	0.85
Q61937	Nucleophosmin	32.56	368	13	0.39
Q9Z204	Heterogeneous nuclear ribonucleoproteins C1/C2	34.385	366	15	0.87
Q62151	Advanced glycosylation end product-specific receptor	42.669	338	6.5	0.29
P62320	Small nuclear ribonucleoprotein Sm D3	13.916	334	17.5	1.1
O08553	Dihydropyrimidinase-related protein 2	62.278	331	10.8	0.41

Table 2 (continued).

P28654	Decorin	39.809	327	9.9	0.31
Q9EST5	Acidic leucine-rich nuclear phosphoprotein 32 family member B	31.079	312	10.3	0.58
Q9EQU5	Protein SET	33.378	309	9.3	0.24
Q9JHL1	Na(+)/H(+) exchange regulatory cofactor NHE-RF2	37.403	304	8	0.21
P62305	Small nuclear ribonucleoprotein E	10.804	300	29.3	0.87
Q60972	Histone-binding protein RBBP4	47.656	292	11.8	0.35
Q9CXW4	60S ribosomal protein L11	20.252	258	19.7	0.68
P16110	Galectin-3	27.515	255	15.2	0.47
P42669	Transcriptional activator protein Pur-alpha	34.884	253	11.2	0.23
P16858	Glyceraldehyde-3-phosphate dehydrogenase	35.81	241	3.6	0.1
P97351	40S ribosomal protein S3a	29.885	238	37.1	1.04
P32067	Lupus La protein homolog	47.756	236	8.9	0.25
P70441	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	38.6	230	5.9	0.2
P01942	Hemoglobin subunit alpha	15.085	229	21.8	0.99
P68433	Histone H3.1	15.404	221	20.6	0.96
P97315	Cysteine and glycine-rich protein 1	20.583	208	7.8	0.18

Table 2 (continued).

Q05793	Basement membrane-specific heparan sulfate proteoglycan core protein	398.294	203	1.1	0.04
P62862	40S ribosomal protein S30	6.648	203	18.6	1.66
P97822	Acidic leucine-rich nuclear phosphoprotein 32 family member E	29.622	198	9.6	0.61
P68369	Tubulin alpha-1A chain	50.136	188	8.6	0.15
Q8VEK3	Heterogeneous nuclear ribonucleoprotein U	87.918	187	8.5	0.23
Q62465	Synaptic vesicle membrane protein VAT-1 homolog	43.097	185	7.9	0.28
P62309	Small nuclear ribonucleoprotein G	8.496	179	17.1	0.48
Q61074	Protein phosphatase 1G	58.728	172	4.4	0.06
P24369	Peptidyl-prolyl cis-trans isomerase B	23.713	166	7.9	0.35
P28656	Nucleosome assembly protein 1- like 1	45.345	163	6.9	0.17
P62307	Small nuclear ribonucleoprotein F	9.725	160	24.4	1
Q8BP67	60S ribosomal protein L24	17.779	158	8.3	0.22
P63325	40S ribosomal protein S10	18.916	155	11.5	0.45
O54724	Polymerase I and transcript release factor	43.954	152	13.3	0.28
P16254	Signal recognition particle 14 kDa protein	12.51	151	21.8	0.72
Q9CR57	60S ribosomal protein L14	23.564	150	6	0.16

Table 2 (continued).

P10126	Elongation factor 1-alpha 1	50.114	147	4.3	0.15
P27048	Small nuclear ribonucleoprotein-associated protein B	23.656	145	9.5	0.56
P57776	Elongation factor 1-delta	31.293	143	7.8	0.25
Q91VM5	Heterogeneous nuclear ribonucleoprotein G-like 1	42.162	141	8.5	0.19
Q9WVE8	Protein kinase C and casein kinase substrate in neurons protein 2	55.833	139	7	0.14
P26040	Ezrin	69.407	134	2.9	0.05
Q9CQI7	U2 small nuclear ribonucleoprotein B"	25.323	133	9.3	0.32
P62702	40S ribosomal protein S4, X isoform	29.598	132	9.9	0.43
Q9DBR7	Protein phosphatase 1 regulatory subunit 12A	114.996	119	3.6	0.1
Q922U2	Keratin, type II cytoskeletal 5	61.767	116	3.3	0.06
P02104	Hemoglobin subunit epsilon-Y2	16.137	116	6.8	0.24
Q8CCK0	Core histone macro-H2A.2	40.092	115	2.4	0.09
Q62093	Serine/arginine-rich splicing factor 2	25.476	114	3.6	0.15
P24549	Retinal dehydrogenase 1	54.468	113	2	0.07
Q7TMK9	Heterogeneous nuclear ribonucleoprotein Q	69.633	113	1.3	0.05
A2ASQ1	Agrin	207.539	107	0.6	0.02

Table 2 (continued).

P47911	60S ribosomal protein L6	33.51	105	9.5	0.38
Q8C4U3	Secreted frizzled-related protein 1	35.413	102	12.4	0.22
P61255	60S ribosomal protein L26	17.258	100	17.2	0.84
Q9DCT8	Cysteine-rich protein 2	22.727	99	10.6	0.16
Q9D0J8	Parathymosin	11.43	98	11.9	0.35
O55126	Protein NipSnap homolog 2	32.933	97	3.9	0.11
P29341	Polyadenylate-binding protein 1	70.671	96	3.1	0.05
Q8BH43	Wiskott-Aldrich syndrome protein family member 2	54.074	92	3.6	0.14
P10649	Glutathione S-transferase Mu 1	25.97	91	8.7	0.31
P08074	Carbonyl reductase [NADPH] 2	25.958	89	4.5	0.15
P20152	Vimentin	53.688	89	6.4	0.07
O09118	Netrin-1	67.81	87	4.5	0.05
Q63918	Serum deprivation-response protein	46.764	81	9.3	0.17
Q8VE97	Serine/arginine-rich splicing factor 4	55.979	80	1.8	0.07
P57784	U2 small nuclear ribonucleoprotein A'	28.357	80	4.3	0.13
Q62376	U1 small nuclear ribonucleoprotein 70 kDa	51.992	76	1.6	0.07

Table 2 (continued).

Q3TEA8	Heterochromatin protein 1-binding protein 3	60.867	75	6.7	0.19
P84104	Splicing factor, arginine/serine-rich 3	19.33	73	5.5	0.2
P61358	60S ribosomal protein L27	15.798	69	5.9	0.25
P62245	40S ribosomal protein S15a	14.84	66	20	0.59
P50404	Pulmonary surfactant-associated protein D	37.688	62	2.1	0.1
P35700	Peroxiredoxin-1	22.176	60	5.5	0.17
P12970	60S ribosomal protein L7a	29.977	59	4.1	0.13
P30115	Glutathione S-transferase A3	25.361	58	10.9	0.32
P14152	Malate dehydrogenase, cytoplasmic	36.511	55	3.6	0.1
O35295	Transcriptional activator protein Pur-beta	33.901	51	5.2	0.23
P47955	60S acidic ribosomal protein P1	11.475	51	14	0.34
Q6ZWN5	40S ribosomal protein S9	22.591	51	4.1	0.17
Q7TNV0	Protein DEK	43.159	50	1.6	0.09
Q3UPL0	Protein transport protein Sec31A	133.569	50	0.6	0.03
P48036	Annexin A5	35.752	49	6.3	0.11
Q9JKB3	DNA-binding protein A	38.814	45	2.2	0.1

Table 2 (continued).

Q8CI43	Myosin light chain 6B	22.749	44	6.3	0.17
P47738	Aldehyde dehydrogenase, mitochondrial	56.538	44	1.9	0.07
Q8R1B4	Eukaryotic translation initiation factor 3 subunit C	105.531	43	1	0.03
P97447	Four and a half LIM domains protein 1	31.889	42	5.4	0.11
Q9CQV8	14-3-3 protein beta/alpha	28.086	41	6.5	0.13
Q9CWL8	Beta-catenin-like protein 1	64.98	41	1.4	0.06
P15532	Nucleoside diphosphate kinase A	17.208	41	13.8	0.22
P15626	Glutathione S-transferase Mu 2	25.717	41	3.2	0.15
P60867	40S ribosomal protein S20	13.373	40	16	0.29
P40630	Transcription factor A, mitochondrial	27.988	37	6.2	0.13
P47915	60S ribosomal protein L29	17.587	35	5.6	0.22
RAGE KO, HDM extract-treated					
P09405	Nucleolin	76.723	2168	19.8	0.94
P28653	Biglycan	41.639	1604	19.5	0.82
Q6GSS7	Histone H2A type 2-A	14.095	1459	43.8	1.09
Q8VCC9	Spondin-1	90.821	1292	24.4	0.66

Table 2 (continued).

P68372	Tubulin beta-2C chain	49.831	1276	25.2	1.05
Q8CGP5	Histone H2A type 1-F	14.162	1267	30	1.08
Q64475	Histone H2B type 1-B	13.952	1221	31.7	1.69
P62082	40S ribosomal protein S7	22.127	1098	34	2.06
P62204	Calmodulin	16.838	1053	41.6	1.29
Q9CZX8	40S ribosomal protein S19	16.085	1033	46.2	6.01
P43274	Histone H1.4	21.977	973	22.4	1.62
P43277	Histone H1.3	22.1	963	22.6	2.06
P67984	60S ribosomal protein L22	14.759	952	40.6	2.24
Q7TMM9	Tubulin beta-2A chain	49.907	827	17.1	0.54
Q61937	Nucleophosmin	32.56	798	14.7	0.55
P60710	Actin, cytoplasmic 1	41.737	794	22.1	0.82
P62751	60S ribosomal protein L23a	17.695	773	16	0.81
P62889	60S ribosomal protein L30	12.784	725	41.7	2.76
P32067	Lupus La protein homolog	47.756	561	13	0.35
O35381	Acidic leucine-rich nuclear phosphoprotein 32 family member A	28.538	470	21.5	0.64

Table 2 (continued).

Q62151	Advanced glycosylation end product-specific receptor	42.669	464	9.2	0.18
P97352	Protein S100-A13	11.158	403	19.4	0.85
P62900	60S ribosomal protein L31	14.463	403	24.8	1.61
Q6ZWV7	60S ribosomal protein L35	14.553	401	22	0.61
P28654	Decorin	39.809	396	9.9	0.31
P62806	Histone H4	11.367	390	34	3.51
Q9Z204	Heterogeneous nuclear ribonucleoproteins C1/C2	34.385	361	12.5	0.87
P68369	Tubulin alpha-1A chain	50.136	347	9.3	0.24
P63017	Heat shock cognate 71 kDa protein	70.871	341	10.7	0.23
P62320	Small nuclear ribonucleoprotein Sm D3	13.916	316	15.1	0.64
P62305	Small nuclear ribonucleoprotein E	10.804	314	29.3	0.87
P62852	40S ribosomal protein S25	13.742	302	20.8	0.65
P16254	Signal recognition particle 14 kDa protein	12.51	301	12.7	0.72
P47962	60S ribosomal protein L5	34.401	282	9.4	0.23
P16110	Galectin-3	27.515	275	5.7	0.14
P97351	40S ribosomal protein S3a	29.885	271	25	1.29

Table 2 (continued).

P97315	Cysteine and glycine-rich protein 1	20.583	246	7.8	0.18
Q9EQU5	Protein SET	33.378	245	9.7	0.38
Q9EST5	Acidic leucine-rich nuclear phosphoprotein 32 family member B	31.079	243	9.9	0.26
P16858	Glyceraldehyde-3-phosphate dehydrogenase	35.81	223	3.6	0.1
P62862	40S ribosomal protein S30	6.648	218	18.6	1.66
P62309	Small nuclear ribonucleoprotein G	8.496	218	17.1	0.48
O54724	Polymerase I and transcript release factor	43.954	215	7.1	0.18
Q922U2	Keratin, type II cytoskeletal 5	61.767	215	2.1	0.06
P97822	Acidic leucine-rich nuclear phosphoprotein 32 family member E	29.622	205	11.9	0.27
Q61074	Protein phosphatase 1G	58.728	199	4.8	0.13
P24369	Peptidyl-prolyl cis-trans isomerase B	23.713	174	7.9	0.35
P28656	Nucleosome assembly protein 1-like 1	45.345	171	4.3	0.08
P26040	Ezrin	69.407	163	4.1	0.11
Q8BP67	60S ribosomal protein L24	17.779	156	8.3	0.22
P10126	Elongation factor 1-alpha 1	50.114	145	4.3	0.15
P47911	60S ribosomal protein L6	33.51	139	9.5	0.24

Table 2 (continued).

Q60972	Histone-binding protein RBBP4	47.656	134	9.2	0.25
Q9CXW4	60S ribosomal protein L11	20.252	133	12.9	0.68
P62702	40S ribosomal protein S4, X isoform	29.598	133	7.2	0.27
P27048	Small nuclear ribonucleoprotein-associated protein B	23.656	133	9.5	0.56
P61255	60S ribosomal protein L26	17.258	128	16.6	0.84
P68433	Histone H3.1	15.404	127	13.2	0.56
P70441	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	38.6	124	3.1	0.1
P10922	Histone H1.0	20.861	123	8.8	0.4
O08583	THO complex subunit 4	26.94	122	11.4	0.3
P29341	Polyadenylate-binding protein 1	70.671	115	3.5	0.11
Q62093	Serine/arginine-rich splicing factor 2	25.476	115	3.6	0.15
P63325	40S ribosomal protein S10	18.916	111	5.5	0.2
P02104	Hemoglobin subunit epsilon-Y2	16.137	108	6.8	0.24
O55126	Protein NipSnap homolog 2	32.933	100	3.9	0.11
Q7TMK9	Heterogeneous nuclear ribonucleoprotein Q	69.633	100	1.3	0.05
Q9CR57	60S ribosomal protein L14	23.564	97	6	0.16

Table 2 (continued).

P24549	Retinal dehydrogenase 1	54.468	94	2	0.07
P20029	78 kDa glucose-regulated protein	72.422	85	2.1	0.05
Q8C4U3	Secreted frizzled-related protein 1	35.413	85	7.3	0.1
A2ASQ1	Agrin	207.539	82	0.6	0.02
P61358	60S ribosomal protein L27	15.798	82	5.9	0.25
P47955	60S acidic ribosomal protein P1	11.475	80	14	0.34
P50404	Pulmonary surfactant-associated protein D	37.688	76	2.1	0.1
P62307	Small nuclear ribonucleoprotein F	9.725	71	9.3	0.41
P84104	Splicing factor, arginine/serine-rich 3	19.33	69	5.5	0.2
Q3UPL0	Protein transport protein Sec31A	133.569	69	0.6	0.03
P10649	Glutathione S-transferase Mu 1	25.97	67	8.7	0.31
Q8VE97	Serine/arginine-rich splicing factor 4	55.979	65	1.8	0.07
P01942	Hemoglobin subunit alpha	15.085	64	11.3	0.58
P12970	60S ribosomal protein L7a	29.977	63	4.1	0.13
Q63918	Serum deprivation-response protein	46.764	62	6.2	0.17
Q8CCK0	Core histone macro-H2A.2	40.092	61	2.4	0.09

Table 2 (continued).

Q62376	U1 small nuclear ribonucleoprotein 70 kDa	51.992	60	1.6	0.07
Q8BH43	Wiskott-Aldrich syndrome protein family member 2	54.074	59	1.6	0.07
P07901	Heat shock protein HSP 90-alpha	84.788	58	1.6	0.04
P18760	Cofilin-1	18.56	57	8.4	0.21
Q9QZZ6	Dermatopontin	23.995	56	5.5	0.16
Q9JKB3	DNA-binding protein A	38.814	55	2.2	0.1
P62245	40S ribosomal protein S15a	14.84	54	20	0.26
P08113	Endoplasmin	92.476	53	1	0.04
P40630	Transcription factor A, mitochondrial	27.988	49	6.2	0.13
P47738	Aldehyde dehydrogenase, mitochondrial	56.538	48	1.9	0.07
Q8VEK3	Heterogeneous nuclear ribonucleoprotein U	87.918	46	2	0.09
Q62465	Synaptic vesicle membrane protein VAT-1 homolog	43.097	44	4.9	0.09
Q3TEA8	Heterochromatin protein 1-binding protein 3	60.867	41	3.2	0.06
Q9R0Q7	Prostaglandin E synthase 3	18.721	38	4.4	0.2
P14211	Calreticulin	47.995	37	1.7	0.08
P63101	14-3-3 protein zeta/delta	27.771	36	7.8	0.14

Table 2 (continued).

O08553	Dihydropyrimidinase-related protein 2	62.278	36	3.1	0.06
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Table 3. Comparison of normalized emPAI values of RAGE binding partners in HDM extract-treated vs. saline-treated WT or RAGE KO mice.

Accession No.	Protein Name	Normalized emPAI (HDM extract-treated) > normalized emPAI (saline-treated)? (Y/N)	
		WT	RAGE KO
A2ASQ1	Agrin	N	Y
A2BDX3	Adenylyltransferase and sulfurtransferase MOCS3	Y	N
O08583	THO complex subunit 4	N	Y
O35381	Acidic leucine-rich nuclear phosphoprotein 32 family member A	N	Y
O54724	Polymerase I and transcript release factor	Y	Y
O55126	Protein NipSnap homolog 2	N	Y
P02104	Hemoglobin subunit epsilon-Y2	Y	Y
P07901	Heat shock protein HSP 90-alpha	N	Y
P08113	Endoplasmin	N	Y
P09405	Nucleolin	Y	N

Table 3 (continued).

P10126	Elongation factor 1-alpha 1	Y	Y
P10649	Glutathione S-transferase Mu 1	N	Y
P12970	60S ribosomal protein L7a	N	Y
P14211	Calreticulin	N	Y
P16110	Galectin-3	Y	N
P16254	Signal recognition particle 14 kDa protein	Y	Y
P16858	Glyceraldehyde-3-phosphate dehydrogenase	Y	Y
P18760	Cofilin-1	N	Y
P20029	78 kDa glucose-regulated protein	N	Y
P24369	Peptidyl-prolyl cis-trans isomerase B	Y	Y
P24549	Retinal dehydrogenase 1	N	Y
P26040	Ezrin	Y	Y
P27048	Small nuclear ribonucleoprotein-associated protein B	Y	Y
P28653	Biglycan	Y	Y
P28654	Decorin	N	Y
P28656	Nucleosome assembly protein 1-like 1	Y	N

Table 3 (continued).

P29341	Polyadenylate-binding protein 1	N	Y
P32067	Lupus La protein homolog	Y	Y
P40630	Transcription factor A, mitochondrial	N	Y
P43277	Histone H1.3	N	Y
P47738	Aldehyde dehydrogenase, mitochondrial	N	Y
P47911	60S ribosomal protein L6	N	Y
P47955	60S acidic ribosomal protein P1	N	Y
P50404	Pulmonary surfactant-associated protein D	N	Y
P60710	Actin, cytoplasmic 1	N	Y
P61255	60S ribosomal protein L26	N	Y
P61255	60S ribosomal protein L26	N	Y
P61358	60S ribosomal protein L27	N	Y
P62082	40S ribosomal protein S7	Y	Y
P62204	Calmodulin	Y	Y
P62305	Small nuclear ribonucleoprotein E	Y	Y
P62307	Small nuclear ribonucleoprotein F	Y	N

Table 3 (continued).

P62309	Small nuclear ribonucleoprotein G	Y	Y
P62315	Small nuclear ribonucleoprotein Sm D1	Y	N
P62320	Small nuclear ribonucleoprotein Sm D3	Y	N
P62702	40S ribosomal protein S4, X isoform	Y	Y
P62737	Actin, aortic smooth muscle	Y	N
P62751	60S ribosomal protein L23a	Y	N
P62806	Histone H4	N	Y
P62843	40S ribosomal protein S15	Y	N
P62852	40S ribosomal protein S25	Y	N
P62862	40S ribosomal protein S30	Y	Y
P62900	60S ribosomal protein L31	Y	N
P63017	Heat shock cognate 71 kDa protein	Y	N
P63101	14-3-3 protein zeta/delta	N	Y
P63325	40S ribosomal protein S10	Y	N
P67984	60S ribosomal protein L22	Y	Y
P68369	Tubulin alpha-1A chain	Y	Y

Table 3 (continued).

P68372	Tubulin beta-2C chain	Y	Y
P84104	Splicing factor, arginine/serine-rich 3	Y	Y
P97315	Cysteine and glycine-rich protein 1	Y	Y
P97351	40S ribosomal protein S3a	Y	Y
P97352	Protein S100-A13	N	Y
P99024	Tubulin beta-5 chain	Y	N
Q3UPL0	Protein transport protein Sec31A	Y	Y
Q60972	Histone-binding protein RBBP4	Y	Y
Q61074	Protein phosphatase 1G	N	Y
Q61937	Nucleophosmin	Y	Y
Q62093	Serine/arginine-rich splicing factor 2	Y	Y
Q62151	Advanced glycosylation end product-specific receptor	Y	Y
Q62376	U1 small nuclear ribonucleoprotein 70 kDa	Y	Y
Q63918	Serum deprivation-response protein	Y	Y
Q64475	Histone H2B type 1-B	Y	N
Q6GSS7	Histone H2A type 2-A	N	Y

Table 3 (continued).

Q6Z WV7	60S ribosomal protein L35	N	Y
Q7TMK9	Heterogeneous nuclear ribonucleoprotein Q	Y	Y
Q7TMM9	Tubulin beta-2A chain	N	Y
Q8BP67	60S ribosomal protein L24	Y	Y
Q8CCK0	Core histone macro-H2A.2	N	Y
Q8CGP5	Histone H2A type 1-F	N	Y
Q8VCC9	Spondin-1	Y	Y
Q8VE97	Serine/arginine-rich splicing factor 4	N	Y
Q8VEK3	Heterogeneous nuclear ribonucleoprotein U	Y	N
Q922U2	Keratin, type II cytoskeletal 5	Y	Y
Q9CR57	60S ribosomal protein L14	N	Y
Q9CXW4	60S ribosomal protein L11	Y	Y
Q9CZX8	40S ribosomal protein S19	Y	Y
Q9EQU5	Protein SET	Y	Y
Q9JKB3	DNA-binding protein A	N	Y
Q9QZZ6	Dermatopontin	N	Y

Table 3 (continued).

Q9R0Q7	Prostaglandin E synthase 3	N	Y
Q9Z204	Heterogeneous nuclear ribonucleoproteins C1/C2	Y	Y

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